AD_____

Award Number: DAMD17-01-1-0160

TITLE: Metastatic Progression of Breast Cancer by Allelic Loss on Chromosome 18q21

PRINCIPAL INVESTIGATOR: Sam Thiagalingam, Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, Massachusetts 02118

REPORT DATE: September 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual Summary (1 Sep 01 - 31 Aug 02)

4. TITLE AND SUBTITLE
Metastatic Progression of Breast Cancer by Allelic Loss on Chromosome 18q21

5. FUNDING NUMBERS
DAMD17-01-1-0160

6. AUTHOR(S):
Sam Thiagalingam, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University
Boston, Massachusetts 02118

E-Mail: samthia@bu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The majority of breast cancer cases is of sporadic origin and is usually diagnosed at an advanced stage. Mutations in the *Smad2* and *Smad4* genes, localized to chromosome 18q, a region frequently deleted in advanced cancers is rare in breast cancer unlike pancreatic, colon, lung and ovarian cancers. These findings support the existence of other tissue specific genes that are specifically targeted for inactivation in breast cancer. Our survey of the various *Smad* genes using a novel technique known as "TEGD" (targeted expressed gene display) has provided the first clues in identifying the *Smad8* gene as an important target for loss of expression in nearly 30% of breast cancers, a level of alteration similar to that of the *HER/neu* gene, a celebrated tumor marker for breast cancer. These studies further suggest that loss of expression of the *Smad4* gene or over-expression of the *Smad7* gene could be important in breast cancer. Our data is consistent with the hypothesis that disabling the Smad signaling pathway could be an important step in the genesis of breast cancer. Further studies to unravel the other modes of alterations of *Smad* genes as well as alternate targets in the Smad signaling pathways are in progress.

14. SUBJECT TERMS
Smad, signaling, metastasis, breast cancer, TEGD, chromosome 18, TGFβ

15. NUMBER OF PAGES
50

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

ANNUAL REPORT OF THE USAMRMC FUNDED ACTIVITY

Title of the grant: Metastatic progression of breast cancer by allelic loss on chromosome 18q21.

1. Introduction/ Project Overview/ Scientific Progress and future directions:

The majority of molecular genetic studies on breast cancer have focused on familial predisposition and there has been a lack of serious effort to understand the molecular basis of the involvement of genetic determinants in the progression to metastatic cancer. The fact that 18q loss has been associated with the advanced carcinoma stage of other cancers suggests that the genes inactivated by this alteration in breast cancer could also be potentially associated with the conversion of benign tumors to malignancy and metastatic progression of the cancer. Unlike pancreatic, colon, lung and ovarian cancers, the lack of mutations in breast cancer in the *Smad2* and *Smad4* genes, localized to chromosome 18q supports the existence of tissue specific genes that are specifically targeted for inactivation in breast cancer and the urgent need for their identification.

Disabling Smad signaling in cancer has become increasingly recognized as an important step that affects processes such as loss of growth inhibition, promotion of angiogenesis and metastasis and the epithelial mesenchymal transition (1). Although significant progress has been made in elucidating the association between genetic alterations in the *Smad4* gene and cancer, the nature of defects involving the other Smads has been elusive, potentially due to alternative mechanisms or targets that result in the loss of or altered signaling end effects. Our survey of the various *Smad* genes has provided the first clues in identifying the *Smad8* gene as an important target for loss of expression in nearly 30% of breast cancers. The epigenetic alterations that underlie these overall abnormalities in signaling could occur at the level of regulation of gene expression or processing of the transcripts. We believe that it is a significant finding as even the most celebrated tumor marker, *HER/neu* gene amplification, also occurs in about 20%-30% breast cancer cases. We hypothesize that *Smad* signaling downstream of the BMPs involving *Smad8* could be an important pathway in breast tissue and inactivation or loss of *Smad8* is a critical tissue specific event in breast tumorigenesis.

On the other hand, there is also emerging data, which supports a potential role for signaling events mediated by the *Smad4* gene, localized to 18q21, in the metastatic progression of cancer. Recent studies suggest that disabling Smad4 signaling leads to increased expression of VEGF (vascular endothelial growth factor), a primary regulator of vascular development, which plays a critical role in angiogenesis and metastasis, and decreased expression of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis (2). We therefore hypothesize that normal Smad4 signaling is required to maintain suppression of metastasis, and inactivation of this signaling is a major step in the development of metastatic breast cancer. Chromosome 18q loss is less frequently observed in breast cancer compared to other cancers and the apparent lack of intragenic mutations in the *Smad4* gene suggests that signaling mediated by Smad4 in suppressing metastatic progression of breast cancer may arise primarily from alterations in other key mediators or effectors which participate in the execution of these signaling events. We propose to identify the mediator and effecter genes, which regulate metastatic progression of breast cancer upon inactivation of the Smad4 signaling pathway using appropriate tumor cell lines as well as experimentally developed derivative test and control cell lines as model systems.

2. Original tasks and the rationale for modifications in the experimental approach:

Although our original proposal was to primarily seek alternate target genes that are either localized to chromosome 18q or novel *Smad* genes involved in metastatic breast cancer, we reformulated our original goals to consider alternate modes of inactivation or loss of function of already known Smads as well as the regulatory and/or effector gene products of the central player, Smad4. The apparent lack of additional target genes localized to 18q21 prompted us to expand our focus to consider inactivation of overall Smad4 signaling pathways due to the fact that disruption or unscheduled activation of critical genes mediating these events could also have

similar effect as direct targeting of the *Smad4* gene. The original tasks, modified expanded tasks and the justification for the slight shift in our original aims are as follows:

Task 1. Directed isolation of *SMAD* genes localized to 18q21 as potential target genes.
Expanded Task 1. Determination and identification of genetic and epigenetic alterations in all known and novel *Smad*s as potential target genes.
Rationale: Although the TEGD (targeted expressed gene display) technique developed by us has not revealed any novel *Smad*s to date, it has enabled the successful detection of previously unknown loss- or over-expression of the different *Smad* genes in breast tumors/ cell lines.

Task 2. Positional cloning of genes localized to chromosome 18q21.
Expanded Task 2. Identification of alternate target genes involving the Smad4 signaling pathway.
Rationale: Despite the lack of inactivation of other known/ predicted genes localized to 18q21, the tissue specific inactivation of gene products that either regulate or mediate the effects of Smad4, the central player in the Smad signaling pathways, could lead to similar outcomes as inactivating the *Smad4* gene itself.

Task 3. Evaluation of candidate target genes. This task remains unmodified.

We have made substantial progress toward the identification of alternate targets for inactivation of the Smad signaling pathways in breast cancer. We have also laid the groundwork for the discovery of Smad4 regulatory and responsive genes as alternate targets for inactivation/ activation in the mediation of metastatic breast cancer. Furthermore, we believe that these studies could shed light on the molecular basis of breast cancer metastasis.

3. Body: Procedures and progress report:
Evaluation of *Smad* alterations in breast cancer.

The analysis of genetic alterations in the various *Smad* genes in previous studies led us to conclude that mutations in *Smad2* and *Smad4*, localized to chromosome 18q, are rarely observed in breast cancer (1). Therefore, we decided to survey the differences in overall expression patterns of the various Smads in breast cancer using the TEGD technique. The TEGD analysis suggested to us that loss of expression of *Smad4* and *Smad8* and over-expression of the *Smad7* gene could be major targets for aberrant Smad signaling events in subsets of breast cancers (Figure 1). We decided to further validate these observations and determine the significance of this abnormality in breast cancer using semi quantitative RT-PCR to analyze the expression patterns using appropriate gene specific primer pairs (Figure 2). We plan to extend these studies to a larger set of breast cancer cell lines and tumors derived from various stages of cancer, and also probe for alternate modes of inactivation in addition to the analysis of gene expression. The initial step to determine gene inactivation will again be by semi quantitative RT-PCR (3; Figure 2). The presence or absence of expression, and the splice variants of the various *Smad*s will be determined by this method. Subsequently, initial mutation searches will be conducted by IVSP analysis and by direct sequencing (3). We will also investigate whether the loss of expression of Smads is due to gene silencing by promoter DNA methylation.

Overall, the preliminary data so far suggested to us that over-expression of *Smad7* or loss of expression of the *Smad8* gene could be major targets for aberrant Smad signaling events in subsets of breast cancers (Figures 1 & 2). Based on these preliminary data, we plan to acquire commercially available antibodies or raise them to Smad 7 and Smad 8 and determine the feasibility of conducting

immunohistochemistry (IHC) on control and test cell lines to assess whether over- or under- expression respectively could eventually be applied to tumor samples (not within the scope of this proposal) for diagnostic and or prognostic evaluations.

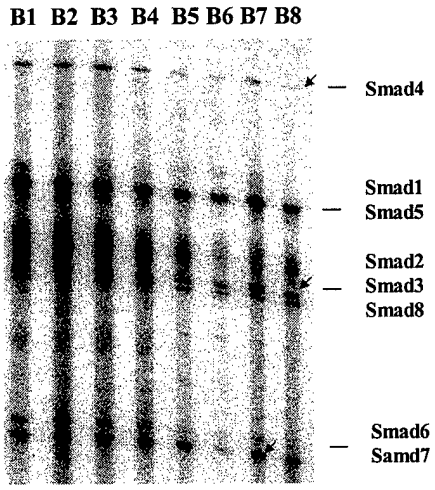


Figure 1. Targeted expressed gene display (TEGD) analysis of Smads in breast cancer.

PCR products for *SMAD*s using degenerate primers were analyzed by TEGD. Lanes B1-8 correspond to PCR products generated using cDNA templates from the normal mammary tissue (B1) and tumor (B2-8) samples. The arrows point to distinct PCR products that were abnormal compared to the normal control. The positions of various Smad genes and their variants as identified from sequence analysis are indicated on the right panel.

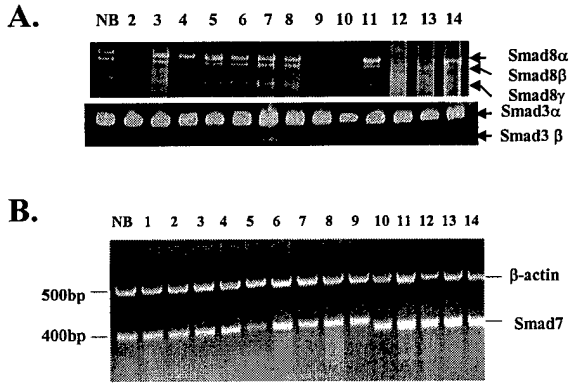


Figure 2. Semi-quantitative RT-PCR analyses of Smad expression in breast cancer.

Total RNA was prepared using the Trizol method from the indicated breast cancer samples and analyzed by RT-PCR (Lanes 1-14). NB refers to sample from normal breast tissue. Smad3α and Smad3β are two of the major differentially spliced forms of Smad3. Smad8α, Smad8β and Smad8γ are three of the major differentially spliced forms of Smad8 which correspond to the full-length, deletion of exon 2, and deletions of exons 2&3, respectively. Analysis of the *β-Actin* gene is used for normalization and quantitation of the *Smad* genes.

Identification and evaluation of Smad4 signaling mediator/ effector genes involved in metastatic breast cancer.

A recent study analyzing the pancreatic adenocarcinoma cell line Hs766T, which harbors a homozygous deletion of the *Smad4* gene, reported an increase in the expression of VEGF and a decrease in expression of TSP-1 (2). These observations suggested that disabling Smad4 signaling events potentially plays a role in promoting the increased propensity for angiogenesis

and metastasis of cancer. We propose to test this phenomenon in breast cancer metastasis. We have chosen the Smad4 inactivated colon cancer cell line HCT116 (*Smad4-/-*) that was experimentally generated to be null for Smad4 and a breast cancer cell line MDA-MB 231, which harbors a deletion of exon 5 in one allele and a point mutation (P303L) in the second allele, thus effectively inactivating the Smad4 gene. We have generated derivative stables, from these Smad4 null/inactive cell lines, that harbor wild-type Smad4 or their corresponding vector controls as the experimental model systems to determine the effect of Smad inactivation in metastatic breast cancer (Figure 3A). The ability to complement Smad4-mediated transactivation in these stable isogenic model cell lines has also been investigated using luciferase reporter assays to confirm the intactness of the Smad4 signaling pathway. These cell lines were transfected with pSBE4-Luc (Luciferase reporter with the Smad4 binding site) to determine whether the expression of Smad4 mediates reporter activity. Our preliminary experiments in which measurements made after 24 hours of culturing with and without TGFβ suggest that Smad4 signaling could be reconstituted (Figure 3C).

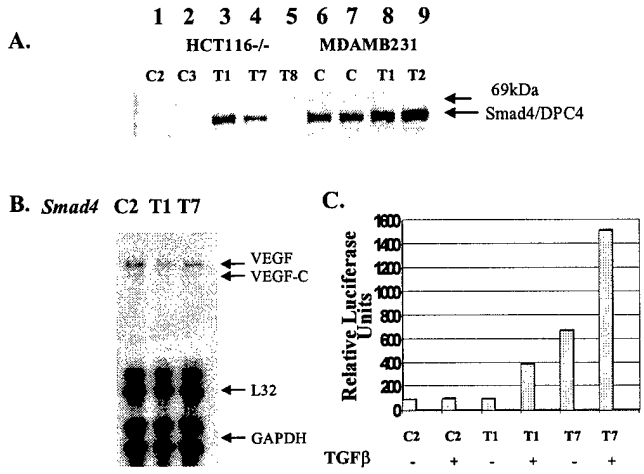


Figure 3. Relationship between *Smad4* status and the expression of VEGF.
A. Western blot analysis to screen for stable Smad4 over-expression in test and control cell lines. Lanes 1&2 and 6&7 are stable transfectants of the pCMV vector while Lanes 3-5 and 8&9 are stable transfectants of the pCMV-*Smad4* of the indicated cell lines. *B. Effect of over-expression of the Smad4 gene on VEGF and VEGF-C.* Total RNA was analyzed with the RiboQuant multi-probe template set (hAngio-1; BD-PharMingen, San Diego, CA) to detect the indicated mRNAs. L32 and GAPDH were included in each template set as internal controls. C. *Smad4 signaling as determined by Smad4 responsive luciferase.* pSBE4-Luc was transfected into the indicated stable cell lines and assessed for Luciferase activity after 24 hours of cell culture.

Furthermore, preliminary experiments are encouraging as the introduction of wild-type Smad4 into the colon cancer cell line HCT116 with the deletion of Smad4 exhibited a decrease in VEGF expression (Figure 3B). The lack of complete suppression of VEGF expression in these experiments could suggest that under the conditions of the experiment the competing pathways that promote VEGF expression could be still operative (e.g., HIF-1α) in these cells despite the fact that Smad4 could function as a major suppressor.

We are also in the process of carefully analyzing our breast cancer models and determining the identities of the various genes that may be differentially regulated and potentially participate in Smad4 mediated processes in the metastatic progression of breast cancer. We plan to undertake the analysis of a wide spectrum of mediator/effector genes/ESTs for their up- or down-regulation using microarrays (Affymetrix).

Once legitimate metastasis mediator and effector gene(s) are identified, evaluation of the

status of the candidate gene(s) for inactivation/ activation in metastatic breast cancer will commence as described in the original proposal (3; Task 3).

4. Key research accomplishments:

Our unexpected preliminary finding that 30% of the breast cancers we sampled exhibited loss of Smad8 expression is very significant as it is equivalent to other established and highly valued markers such as *Her/neu.* The identification of target gene(s) that disable Smad signaling to promote breast cancer could potentially provide key arsenals to combat breast cancer.

We have identified/ generated appropriate tumor cell lines as well as experimentally developed derivative test and control cell lines as model systems to identify and isolate the metastatic breast cancer mediator and effecter genes involving Smad4.

5. Conclusions:

(1) Loss of expression of *Smad4* and *Smad8* and over-expression of the *Smad7* gene are potentially major mechanisms for inactivation of Smad signaling in breast cancer.
(2) The loss of Smad8 expression in 30% of the breast cancers is very significant as it is equivalent to other established and highly valued tumor markers such as *Her/neu.*
(3) The identification of target gene(s) that disable Smad signaling to promote breast cancer could potentially provide not only novel and valuable diagnostic and prognostic tumor markers but also key arsenals to combat breast cancer.

6. References:

1. Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGFβ and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.
2. Schwarte-Waldhoff I, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, and Schmiegel W. 2000. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Pro. Natl. Acad. Sci.* 97: 9624-9629.
3. Thiagalingam, S. 2001. Molecular detection of Smad4/Smad2 alterations in colorectal tumors: Colorectal Cancer Methods and Protocols. In *Methods in Molecular Medicine.* S. M. Powell (Ed) Humana Press Inc., New Jersey, 50: 149-165.

7. Scientific presentations/ publications relevant to this grant:

Seminars by Dr. Sam Thiagalingam:

LOH: A predictor to map tumor suppressor genes involved in cancer and the molecular mechanisms of their occurrence. DDC Seminar, Medical College of Wisconsin, Milwaukee, October 15, 2001.

The Smad connection to cancer. Boston University School of Medicine Rheumatology Conference. September 4, 2002.

Publications:

Cheng, K-h., H. J. Lee, J. F. Ponte and S. Thiagalingam. 2002. Identification of alternate targets for the inactivation of Smad signaling in cancer using a novel method: Targeted Expressed Gene Display. *Manuscript in preparation.*

Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGFβ and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.

Thiagalingam, S., R. L. Foy, K-h.Cheng, H. J. Lee, A. Thiagalingam, and J. F. Ponte. 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. *Current Opinion in Oncology* 14(1): 65-72.

APPENDIX I -Reprints

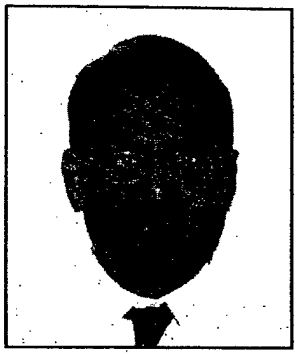
Relevant publications by the P. I.:

1. Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGF-β and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.

2. Thiagalingam, S., R. L. Foy, K-h.Cheng, H. J. Lee, A. Thiagalingam, and J. F. Ponte. 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. *Current Opinion in Oncology* 14(1): 65-72.

TGFβ and its *Smad* Connection to Cancer

S. Thiagalingam*, K-h. Cheng, R. L. Foy, H. J. Lee, D. Chinnappan and J. F. Ponte



Genetics and Molecular Medicine Programs and Pulmonary Center, Department of Medicine and Department of Pathology & Laboratory Medicine, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118, USA

Abstract: The resistance to growth inhibition commonly observed in a variety of TGFβ disabled human cancers, the potential role of TGFβ in the exacerbation of malignancy and the effects of TGFβ in suppressing the immune system, all emphasize the importance of pathways mediated by this polypeptide to the neoplastic process. Early investigations to understand the molecular basis of cancer due to defects in TGFβ signaling were concentrated on examining the abundance of biologically active TGFβ and its binding to TGFβ receptors. However, major breakthroughs in understanding the molecular basis of the TGFβ mediated effects in cancer came from genetic evidence for inactivation of the various players in its signaling cascade. The vast majority of current evidence is derived from the identification of mutations causing structural defects in TGFβ receptors and *Smad* genes, the downstream effectors of the TGFβ signaling pathway that have emerged from the analysis of human cancers. The involvement of Smads at the receptor level upon activation by a TGFβ bound receptor, their participation in signal transmission to the nucleus and their direct roles in the regulation of target genes have made the various *Smad* genes critical targets for inactivation of TGFβ signaling in cancer. To date, eight human homologues of the *Smad* genes have been identified and are classified into three distinct classes based on their structure and function. In this review, we discuss TGFβ signaling *via* the Smads and the known and predicted points at which TGFβ signaling could become altered in human cancer.

INTRODUCTION

The signaling pathways mediated by the members of the transforming growth factor-beta (TGFβ) family are implicated in a number of biological processes including cell differentiation and proliferation, determination of cell fate during embryogenesis, cell adhesion, cell death, angiogenesis, metastasis and immunosuppression [1-4]. Due to the wide array of functional consequences mediated by its signaling events, TGFβ could impact tumorigenesis by affecting any one or a combination of the following processes: (1) altering the delicate balance between cellular proliferation and apoptosis; (2) affecting induction of extracellular matrix proteins such as proteoglycans, collagens, fibronectin, laminin, tenascin and vitronectin and regulating their breakdown by extracellular proteases and metalloproteinases by controlling the induction of their inhibitors such as plasminogen activator inhibitor (PAI-1) and tissue inhibitor of metalloproteinase (TIMP); and (3) disabling the tumoricidal activity or cytokine production of the immune system [5-11]. The major mechanisms that disrupt the carefully regulated balance of these events may consist of changes in the induction of gene expression patterns and in the functionality of proteins, which are affected by internal or external cues or familial and/or sporadic genetic changes. Although there has been significant progress in unraveling some of the genetic and epigenetic alterations that underlie these overall abnormalities in signaling by TGFβ, the nature and cues prompting changes that occur at the level of regulation of gene expression, protein synthesis and/or post-translational modifications will most likely be elucidated only by the efficient application of high throughput methodologies such as gene expression microarrays and proteomics.

TGF-β SIGNALING

Although TGFβ was originally discovered for its positive role in transformation and tumor progression, most of the recent efforts have focused on the understanding of the mechanism of epithelial cell growth inhibition [12-14]. The TGFβ family of factors is comprised of nine subfamilies/ members with homology at the amino acid level ranging from 23 to 92%. The BMP (Bone Morphogenetic Protein), activin and TGFβ subfamilies have been the most widely studied. The TGFβ family members bind to specific receptors, which consist of two major subfamilies, type I (RI) and type II (RII) receptors. These receptors are structurally similar and have cysteine rich extracellular regions and an intracellular kinase domain with serine/ threonine kinase activity [15,16]. The RI receptor has a conserved 30 amino acid segment adjacent to the kinase domain rich in glycine and serines known as the GS region which forms a wedge against the catalytic center [17]. TGFβ factors, which are dimers, bring together the RII and RI receptors into a heterotetrameric complex. The RII receptor's kinase domain becomes constitutively active and phosphorylates the GS domain of the RI receptor. Activated RI receptors can then mediate their activities through the

*Address correspondence to this author at the Genetics and Molecular Medicine Programs, Department of Medicine and Department of Pathology & Laboratory Medicine, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118, USA; Tel: 617-638 6013; Fax: 617-638 4275; E-mail: samthia@bu.edu

1389-2029/02 $35.00+.00

Smad proteins. The role of vertebrate Smad (Sma and Mad) proteins in TGFβ signaling was predicted from their high level of homology to the Mad (Mothers against dpp) from *Drosophila* and the Sma-2, Sma-3 and Sma-4 proteins from *Caenorhabditis elegans* in analogous signaling pathways [18-22].

To date at least eight homologues of the *Smad* genes have been identified and shown to be downstream of the serine/threonine kinase receptors (Table **1**, Fig. **1**). Smads are molecules with a relative mass of 42Kd-60Kd composed of two regions of homology (Mad Homology (MH) domains) at the amino and carboxy terminals of the protein termed the MH1 and MH2 domains, respectively, which are separated by a proline rich acidic linker region of variable length and sequence [34-36]. The amino terminal MH1 domains share 40-94% homology among Smads while the carboxy terminal MH2 domains are 38-98% homologous in their amino acid sequence (Fig. **1**), [35]. The MH2 domain is involved in homo and heteromeric complex formation, as well as in transcriptional activation and repression, whereas the MH1 domain has DNA binding activity [36-38]. The MH2 domain of Smad2 and Smad3 interacts with the RI receptor [39]. Prior to activation of the receptor-regulated Smads by receptors, MH1 and MH2 domains interact with each other resulting in auto-inhibition [40,41].

The Smad family of proteins is divided into three distinct classes based on their structure and function [12]. The first category consists of pathway-restricted or receptor regulated Smads (R-Smads): Smad1, Smad5 and Smad8 (also known as MADH6/Smad9 in humans), which are specifically involved in BMP signaling whereas Smad2 and Smad3 are TGFβ/activin pathway restricted. These Smads are directly phosphorylated by RI receptors after phosphorylation of the RI receptor by the RII receptor. The pathway restricted Smads have a characteristic Ser-Ser-X-Ser (SSXS) motif in their C-terminal region. The two-most C-terminal serine residues of these Smads are phosphorylated by RI receptors [42-44]. Phosphorylation at these sites has been shown to be necessary for Smad2 to interact with the second class of Smads known as the common mediator Smad (Co-Smad) [45]. Smad4 is the only member of this class of Smads known in mammals. However, the recent identification of two Smad4 proteins (Xsmad4α and Xsmad4β) in *Xenopus* opens up the possibility that homologues of Smad4 may exist in mammals [46]. Smad4 lacks the C-terminal SSXS motif and is not phosphorylated by RI receptors [41]. Smad4 is involved in all distinct pathways and plays a central role by forming heteromeric complexes with the R-Smads.

SARA (Smad anchor for receptor activation) is a FYVE domain protein that only interacts with the MH2 domains of Smad2 and Smad3 [47]. SARA exhibits preferential binding to unphosphorylated Smad2 and becomes released when Smad2 is phosphorylated by the TGFβRI receptor. The Smad2-SARA and Smad2-Smad4 complexes apparently exist in a mutually exclusive manner. The double zinc finger FYVE domain of SARA is believed to be involved in directing the localization of R-Smads to the membrane where the TGFβ RI receptors are located and thus increasing the effective concentration of Smad signaling molecules in their vicinity. Once the R-Smad molecules become phosphorylated and released from the SARA adaptor protein, phosphorylated R-Smads form R-Smad-Co-Smad complexes that translocate from the cytoplasm to the nucleus.

The crystal structure of the Smad4 MH2 domain has been resolved [35]. The monomer contains a β-sheet sandwich, capped at one end with a group of three large loops and an α-helix (loop-helix domain) and at the other end by a triple α-helix structure. These monomers assemble to form a trimeric structure in the crystal with the loop helix domain of one monomer interacting with the triple helix of the next; the resulting structure resembles a disk. On the face of the disk opposite the amino terminal side, the third loop (L3) from the loop-helix is exposed on the surface. It has been suggested that this loop is critical in mediating the formation of a hexameric complex between Smad4 trimers and trimers of phosphorylated R-Smads [48,49]. In addition to these predictions based on crystal structures, other models for the nature of Co-Smad and R-Smad hetero-oligomeric complexes have been proposed from biochemical and structural studies, which include heterohexamer, heterotrimer and heterodimer formations. [48-53]. At the time of this writing, the most recent biochemical and functional data

Table 1. Human SMAD Genes and Cancers

Gene	Map position	Alterations in cancers	Reference(s)
SMAD1	4q28-31	Not detected	[23]
SMAD2	18q21	Colon, liver and lung	[23,24,25]
SMAD3	15q21-22	Not detected	[23,26]
SMAD4	18q21	Colon, head & neck, leukemias, liver, lung, ovary and pancreas	[21,23,26-31,151]
SMAD5	5q31	Not detected	[23,26]
SMAD6	15q21-22	Not detected	[23,26]
SMAD7	18q21	Not detected	[23,26,32]
SMAD8	13q12-14	Not detected	[33]

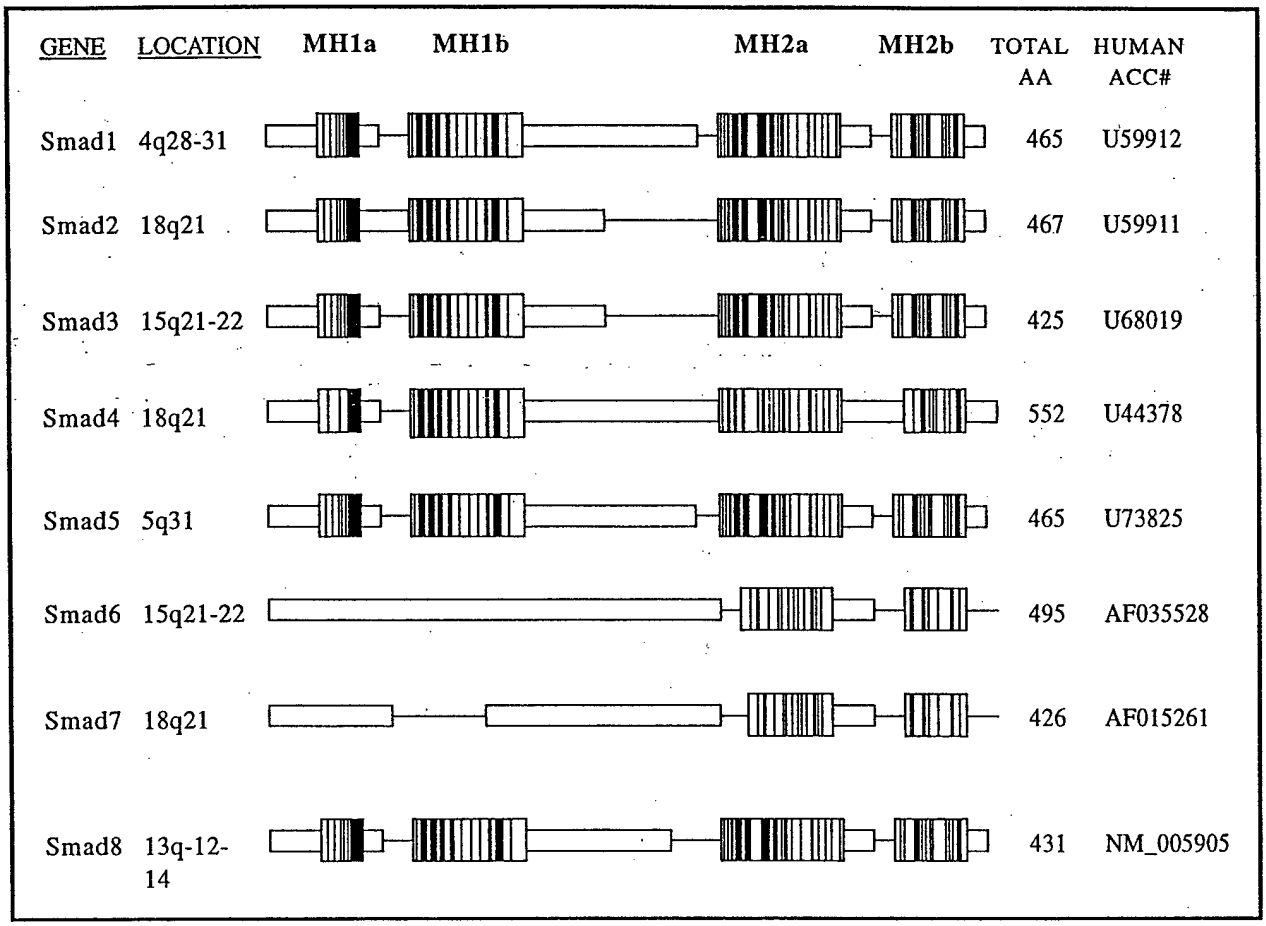


Fig. (1). Amino acid alignment of the known human Smad homologues.

Mad homology (MH) domains, labeled MH1a, MH1b, MH2a and MH2b, represent stretches of sequence that are highly related in Smad proteins. The thin vertical lines denote identical residues in at least five genes. The horizontal lines signify gaps introduced in order to optimize the alignment. Using Smad1 as a reference, MH1a, MH1b, MH2a and MH2b extend from codons 20 to 45, 68 to 145, 265 to 367, and 402 to 454, respectively. The inhibitory Smads, Smad6 and Smad7, do not possess MH1 domains, but the two of them do share regions of homology in their N-termini. The chromosomal position is shown in the second column, and accession numbers for the human *Smad* genes are shown in the last column.

strongly support the existence of a heterodimer between a Co-Smad and a R-Smad as the predominant basic functional unit [52,53].

The structural analysis of the Smad3 MH1 domain showed that the domain adopts a compact globular fold, with four α-helices, six short β strands, and five loops. A β-hairpin loop mediates the direct contact with DNA in the major groove. The β-hairpin loop is comprised of residues Leu-75, Gln-76, and Val-77 of strand B2, residues Arg-80, Lys-81, and Gly-82 of B3, and two connecting residues, Ser-78 and His-79. The β-hairpin is among the most highly conserved regions in Smad proteins and all the residues except the two at the turn of the β-hairpin are invariant among mammalian Smads [35]. Smad3 and Smad4 bind to DNA via the MH1 domain at sequences that contain AGAC, called Smad Binding Elements (SBEs) [55-57]. It has also been shown that an extra stretch of amino acids N-terminal to the β-hairpin loop present in Smad2 (derived from exon 3) affords steric hindrance which inhibits DNA binding of the homo-oligomer of Smad2 [35,55,58].

The regulation of Smad signaling could occur at several levels including direct effects on Smad molecules affecting their functional status or by determining their stability or half-life. Upon translocation into the nucleus, each of the R-Smad-Co-Smad complexes could activate a specific set of genes through cooperative interactions with DNA and other Smad interacting DNA-binding proteins (SIDBP) and/or cofactors. R-Smad translocation to the nucleus is not dependent upon Smad4, but Smad4 translocation into the nucleus requires an activated R-Smad [59,60]. It has been shown that the MH1 and MH2 domains of Smads interact with a number of factors in the nucleus to determine the specificity of gene activation or repression (Table **2**) [61,62]. This specificity for DNA binding of Smad transcription factor complexes to regulatory regions of genes would theoretically lead to transcriptional activation or repression

Table 2. Cofactors of Smad Transcriptional Regulation

Co-Factor	Smad/ Domain Contacted	Molecular Basis of Activity	Reference(s)
Activators			
AML/PEBP2/CBFA	Smad2, 3 MHI, MH2	Cooperate with Smads to induce gene expression	[82-84]
ATF-2	Smad3 MH1	"	[85]
Mixer/milk	Smad2 MH2	"	[86]
Fast1/Fast2	Smad2, 3 MH2	"	[63, 64, 86]
c-Fos	Smad3 MH2	"	[87]
c-Jun, JunB, JunD	Smad3 MH1, Linker	"	[87-89]
SP1	Smad2, 3, 4 MH1 and/or MH2	"	[90-94]
CBP and p300	Smad2, 3 MH2; Smad4 activation domain	Opens chromatin structure by histone acetylation	[55, 57, 88, 95-98]
MSG1	Smad4 MH2	Interacts with CBP and p300	[99, 100]
Repressors			
AML/ETO	Smad2, 3 MH2	Unknown	[101]
SIP1	Smad2, 3 MH2	Unknown	[74, 81]
TGIF	Smad2, MH2	Recruits histone deacetylases	[38, 75]
E1A	Smad3 MH2	Prevents interaction with CBP and p300	[102]
Evi-1	Smad3 MH2	Prevents Smad3 binding to DNA	[103, 104]
Ski, SnoN	Smad2, 3, 4 MH2	Recruit repressors, N-CoR, mSin3 and histone deacetylases. Bind DNA	[37, 77-80]

of a unique set of gene(s) with a high level of specificity for the tissue and/or the overall physiological signal being transduced. The binding of an activated Smad transcription complex to specific regulatory regions and transactivation may be defined in part by subsequent SIDBPs such as FAST1, FAST2 and Jun/Fos [59, 63-67]. On the other hand, transcriptional specificity could also occur by recruitment of factors interacting with Smads by protein-protein interactions such as in the cases of p300 and CBP (CREB-binding protein) [68-71]. p300 and CBP are histone acetyl transferases (HAT) that increase transcriptional activation due to their ability to alter the nucleosome structure *via* chromatin remodeling by hyperacetylation of neighboring chromatin and by recruitment of the RNA polymerase holoenzyme to the promoter [72,73]. Similarly, DNA binding factors such as SIP1 and TGIF or non-DNA binding factors such as Evi1, Ski and SnoN could interact with the Smad proteins in the transcription complex leading to the repression of transcription [74-81]. The homeodomain protein TGIF (TG interacting factor) is believed to mediate Smad-dependent repression of transcription either by recruiting histone deacetylases (HDACs) or by competing with p300 or CBP for association with Smads [1,75]. Alterations in the relative levels of the coactivators or corepressors in the cell depending on the tissue type and/or physiological state would ultimately determine whether TGFβsignaling would result in activation or repression of specific genes.

The third class of Smads includes Smad6 and Smad7 which were identified as anti-Smads or inhibitory Smads (I-Smad) due to their ability to act as inhibitors of TGFβ signaling. Smad6 and Smad7 are rapidly induced by TGFβ-like molecules, IFNγ, and a number of other growth factors and cytokines and they elicit negative feedback regulation of Smad signaling pathways. Smad6 and Smad7 interact with activated RI receptors with high affinity to prevent access to RI receptors by R-Smads and thus inhibit phosphorylation of R-Smads [32,105,106]. STRAP (serine-threonine kinase receptor associated protein) contains a WD domain and associates with Smad7 as well as with the RI receptors augmenting the inhibitory effects of Smad7 [90,107]. Furthermore, it has been postulated that Smad6 may also compete with Smad4 for association with activated Smad1 in BMP signaling, and may possibly function as a co-repressor for transcription *via* its interaction with Hoxc-8, a homeodomain protein factor [108].

The ubiquitin proteosome pathway is implicated in the turnover of Smads as well as TGFβ receptors in the regulation of the TGFβ signaling pathway. Smurf1 and Smurf2, E3-ubiquitin protein ligases, target R-Smads of the BMP signaling pathway (Smads 1 and 5), Smad7 and TGFβ receptors respectively [109,110]. Smad7 expression is stimulated by IFN-γ and promotes Smurf2-Smad7 complex formation in the nucleus; this complex is translocated to the cytoplasm and eventually binds to the TGFβ receptors at the

plasma membrane leading to their degradation [110]. Poly-ubiquitination of Smad2 and subsequent degradation by the proteasome has also been reported suggesting that regulation of the TGFβ signaling components occur through the ubiquitin proteosome pathway [111].

Additional detailed accounts of Smad signaling can also be found in some of these recent reviews [2,3,112,113]. A heuristic model illustrating the different roles of the Smads in TGF-β signaling is outlined in Fig. (2).

ABUNDANCE OF BIOLOGICALLY ACTIVE TGFβ IN CANCER

TGFβigands secreted in their latent forms are approximately 390 to 414 amino acids long and consists of a latent associated peptide (LAP) region at the amino-terminal and the biologically active mature form of TGFβ at the carboxy-terminal ends [114]. Proteolytic processing in the Golgi apparatus by the convertase family of endoproteases and conformational changes assisted by LAP are believed to be necessary to form the biologically active form of TGFβ.

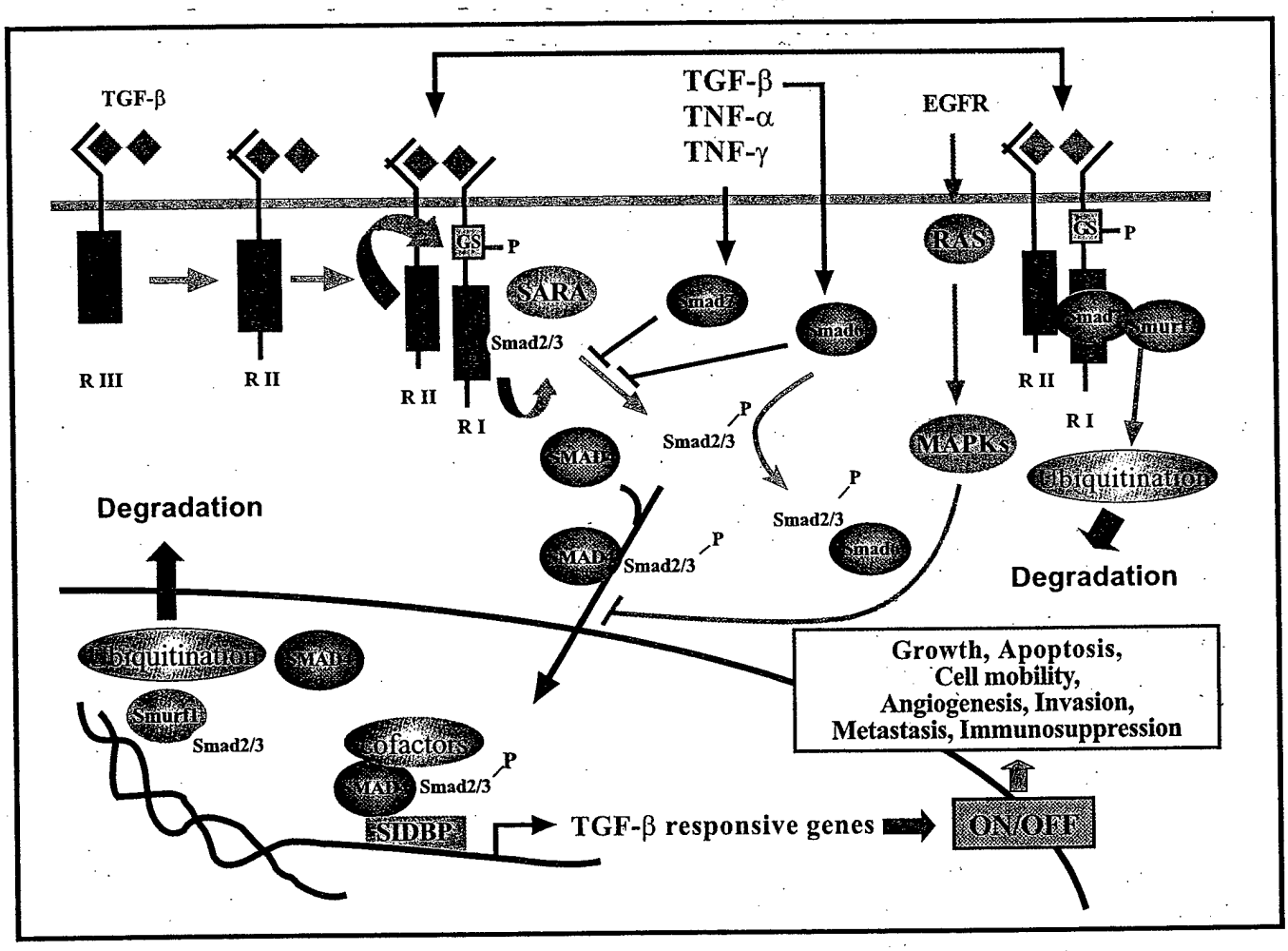


Fig. (2). A model for the Smad connection to the TGFβ signaling pathway.

TGFβ binds to a type-III receptor, which helps to increase the localized effective concentration of the ligand in the proximity of the type-II receptor (RII). The TGFβ ligand binds to the RII which phosphorylates a type I receptor (RI) that in turn initiates signaling *via* the Smad proteins. The activated RI recognizes receptor regulated Smads (R-Smad), such as Smad2 or Smad3 and phosphorylates them at specific carboxy-terminal serine residues. These phosphorylated R-Smads form a heteromeric complex with the common-mediator Smad (Co-Smad), Smad 4, and the complex is translocated into the nucleus. In the nucleus, the R-Smad/ Co-Smad hetero-oligomer, either by itself or by associating with heterologous Smad interacting DNA binding proteins (SIDBPs), such as FAST-1, and/or other cofactors, mediate specific transcriptional activation or repression responses. The inhibitory Smads (I-Smads), such as Smad6 and Smad7, are able to compete with R-Smads by stably binding the RI or by preventing association of R-Smads with Co-Smads, effectively blocking downstream signaling events. There are numerous other signaling pathways such as Ras-MEK that could modulate the end effects by establishing cross talk among the different pathways. The ubiquitin-proteosome pathway continuously recycles the various players in the TGFβ signaling pathway, thus contributing to the inducibility of the system. Please refer to the text for more details.

LAP remains noncovalently linked to the bioactive TGFβ and the TGFβ-LAP complex is apparently bound by latent TGFβ binding proteins (LTBP) to stabilize the protein, ensure correct folding of TGFβ and to enhance secretion of TGFβ to the extracellular matrix of target cells for storage or bioactivity. It has been reported that LTBP was absent in malignant but not in benign prostatic tissue suggesting that tumorigenecity promoted by TGFβ could be regulated at the ligand level [115]. The other molecules, which could play critical roles in determining the levels of free and mature forms of TGF-β available to initiate signaling, are thrombospondin-1 (TSP-1), endoglin (CD105) and betaglycan (TGFβ receptor type III (TGFβ R III)). TSP-1 is suggested to play an important role in the activation of TGFβ by inducing conformational changes in LAP, which prevents reassociation between LAP and mature TGFβ [116,117]. Endoglin exhibits strong homology to betaglycan in the transmembrane and intracellular domains and binds to TGFβ. It is reported to be overexpressed in breast and lung cancers with increased angiogenesis and decreased TGFβ responses [118-121]. On the other hand, overexpression of the TGFβ RIII is associated with enhanced TGFβ responsiveness due to its ability to act as a reservoir for active TGFβ ligands. TGFβ RIII has high affinity binding sites for TGFβ ligands and facilitates their interaction with TGFβ RII [122-124]. Under some conditions increased expression of TGFβ RIII has been associated with reduced tumorigenicity, however, it is believed to enhance tumorigenicity under other conditions [121]. The increase in tumorigenicity observed under these latter conditions may be associated with metastatic conversion of tumor cells in advanced cancer.

TGFβ RECEPTOR DEFECTS IN CANCER

Resistance to TGFβ mediated growth inhibition is also found to be common in a variety of human cancers, which emphasizes the importance of pathways mediated by this polypeptide to the neoplastic process [125-127]. The early investigations to understand the molecular basis of this resistance were concentrated at the level of TGFβ RI and RII, the serine/threonine kinase receptors. A correlation between resistance to TGFβ growth inhibition due to lack of TGFβ receptor expression was established and reported in a variety of human cancer cell lines [128-131]. The first genetic evidence for the inactivation of the TGFβ signaling pathway due to mutations causing structural defects in the TGFβ RII revolutionized the understanding of the molecular basis of this defect [129,131,132]. RII mutations were initially reported in colon cancer with microsatellite instability (MSI) resulting from frame shifts clustered in a naturally occurring 10 bp microsatellite-like polyadenine tract in the 5' coding half of the gene. Subsequent studies demonstrated that additional sites such as residues in the kinase domain could also be inactivated in both MSI and non-MSI colon tumors [131-133]. Microsatellite instability in this polyadenine repeat, referred to as the BAT-RII region, leads to frameshift mutations resulting in truncated receptors that lack kinase domains and are thus functionally inactive [131]. In addition to colorectal cancers, mutations in the BAT-RII region are also found in gastric cancers and in gliomas, but rarely in microsatellite instable tumors of the pancreas, liver, endometrium and breast [131,132,134]. Furthermore, most of the non-BAT-RII mutations are centered in the kinase domain and affect the ability of the RII receptor to phosphorylate the RI receptor [133]. The analysis of a mutation, Thr315Met, in the kinase domain of the RII receptor exhibited the separability of end effects mediated by TGFβ signaling, which may be important in cancer. This mutation in HNPCC kindred retained the ability to induce extracellular matrix proteins and PAI-I characteristic of the metastatic potential while it lost its ability to induce p15 leading to the lack of TGFβ mediated growth inhibition [135].

There is also evidence that TGFβ RI could become inactivated in a subset of other cancers [127]. It has been reported that 33% of ovarian cancers have an inactivating mutation in the RI receptor while no mutations were observed in the type II receptor in the same tumor cohort [136]. Additionally, deletions of the RI receptor have been reported to occur at a low frequency in pancreatic and biliary carcinomas as well as in T cell lymphomas while mutations of specific amino acid residues were found in breast cancer [137-139]. A recent analysis of gastric cancer cell lines suggests that silencing of TGFβ RI expression could also occur due to hypermethylation of a CpG island in the promoter region adding another mechanism of regulation that determines the abundance of TGFβ receptors [140]. However these alterations alone do not explain the mechanism of inactiva-tion of the TGF-β signaling pathway in an overwhelming number of tumors that are resistant to TGFβ signaling effects.

INACTIVATION OF SMADS IN CANCER

The recent discovery of the *Smad* genes as downstream effectors of the TGFβ signaling pathway and the frequent occurrence of mutations in these genes could be regarded as a major breakthrough in the understanding of the molecular basis of insensitivity and/or deregulation of TGFβ mediated effects [21,23,27,28,141,142]. The isolation of the *Smad4* gene itself was based on the identification of target tumor suppressor genes localized to frequent homozygous deletions affecting 18q21.1 in pancreatic carcinomas [21]. Allelic loss of 18q21.1 has been associated with cancers of higher mortality and an increased risk of metastatic spread [27,143,144]. In addition to pancreatic cancer, *Smad4* mutations were also found in a subset of colon and lung cancers but rarely in others (Table 3) [23,27,28,141,142]. Two novel *Smad* genes, in addition to *Smad4*, have been isolated and localized to chromosome 18q21 (Table 1) [24,25,32]. Soon after the discovery of *Smad4*, *Smad2* was also localized to 18q21 and considered as a legitimate alternate candidate tumor suppressor gene localized to this region. Mutational analysis of *Smad2* by others and by us revealed that it is also inactivated in additional colorectal tumors [24,25]. In our study, we extended the analysis of *Smad4* inactivation by using the same set of tumors with LOH at 18q21 and found that some of the tumors, which lacked *Smad4* mutations harbored *Smad2* mutations (Table 4) [21,24,142]. These observations further strengthened our hypothesis that these two genes and other genes involved in TGF-β signaling may be alternatively targeted for

Table 3. Mutations in *Smad4*

Codon	Mutation	Predicted Change	Cancer	Reference(s)
43	TTG to TCG	Leu to Ser	Pancreas	[26]
50-51	A insertion	Frameshift/Stop	Colon	[150]
51	AAA to AAG	Lys to Lys (Silent)	Ovary	[151]
65	GGG to GTG	Gly to Val	Colon	[152]
95	TAT to AAT	Tyn to Asn	Colon	[150]
100	AGG to ACG	Arg to Thr	Pancreas	[28]
102	CCT to CTT	Pro to Leu	AML	[153]
115	TGT to CGT	Cys to Arg	Colon	[154]
129	AAT to AAG	Asn to Lys	Colon	[150]
130	GTC to GAC	Pro to Ser	Colon	[142]
162	2bp deletion	Frameshift/ Stop	Pancreas	[26]
168	GGA to TGA	Gly to Stop	Colon	[154]
195	TAC to TAA	Tyr to Stop	Colon, Pancreas	[26,154]
202-203	4bp deletion	Frameshift/ Stop	Lung	[141]
269-270	ACT to ACTT	Frameshift/ Stop	Colon	[154]
317	CAT to CGT	His to Arg	Ovary	[151]
326	1bp deletion	Frameshift/Stop	Colon	[150]
332	GAT to GGT	Asp to Gly	Liver (HCC)	[29]
336-338	2bp deletion	Frameshift/ Stop	Colon (HNPCC)	[154]
339-343	15bp deletion	Frameshift	Colon	[154]
343	TCA to TGA	Ser to Stop	Pancreas	[28]
343	2bp deletion	Frameshift/ Stop	Pancreas	[28]
351	GAT to CAT	Asp to His	Pancreas, Ovary	[28,151]
355	GAC to GAA	Asp to Glu	Colon	[150]
358	GGA to TGA	Gly to Stop	Colon, Pancreas	[21,142]
361	CGC to TGC	Arg to Cys	Colon	[142]
361	CGC to CAC	Arg to His	Colon	[154]
363	TGT to AGT	Cys to Ser	Colon	[154]
369	AAT to GAT	Asn to Asp	Pancreas	[155]
370	GTC to GAC	Val to Asp	Colon	[142]
379	GCA to ACA	His to Arg	Ovary	[151]
386	GGT to TGT	Gly to Cys	Ovary	[151]
401	TGC to CGC	Cys to Arg	Liver (HCC)	[29]
406	GCG to ACG	Ala to Thr	Pancreas	[26]
412	TAC to TAG	Tyr to Stop	Pancreas	[21]
415-416	4bp deletion	Frameshift/Stop	Colon	[154]
420	CGT to CAT	Arg to His	Lung	[141]

(Table 3) contd....

Codon	Mutation	Predicted Change	Cancer	Reference(s)
441	CGT to CCT	Arg to Pro	Lung	[141]
442	CAG to TAG	Gln to Stop	Colon	[154]
445	CGA to TGA	Arg to Stop	Colon	[150,154]
447-455	25bp deletion	Frameshift/Stop	Colon	[154]
450-459	28bp deletion	Frameshift/Stop	Colon	[154]
457	GCA to TCA	Ala to Ser	Pancreas	[155]
483	AGT to AAT	Aberrant splicing	Pancreas	[21]
483	4bp insertion	Frameshift/Stop	AML	[153]
492	1bp insertion	Frameshift/Stop	Seminoma germ cell	[30]
493	GAT to CAT	Asp to His	Pancreas	[21]
497	CGC to CAC	Arg to His	Colon	[154]
504	AGT to AGA	Ser to Arg	Ovary	[151]
507	AAA to CAA	Lys to Gln	Colon	[154]
515	AGA to GGA	Arg to Gly	Colon	[154]
515	AGA to TGA	Arg to Stop	Pancreas	[21,155]
516	CAG to TAG	Gln to Stop	Colitis	[156]
516-518	8bp deletion	Frameshift/ Stop	Pancreas	[21]
525	ATT to GTT	Ile to Val	Head and Neck	[157]
526	GAA to TAA	Glu to Stop	Head and Neck	[157]
528/529	4bp deletion	Frameshift/ Stop	Pancreas	[155]
538	GAA to TAA	Glu to stop	Colon	[150]
540-542	7bp deletion	Frameshift/ Stop	Colon	[154]

Table 4. Mutations in *Smad2*

Codon	Mutation	Predicted change	Cancer	Reference(s)
133	CGC to TGC	Arg to Cys	Colon	[25]
345-358	42bp in deletion	In frame deletion	Colon	[24]
346	TTT to GTT	Phe to Val	Colon	[154]
407	CAG to CGG	Gln to Arg	Liver (HCC)	[29]
431-454	9bp in deletion	In frame deletion	Lung	[27]
441	CAT to CGT	His to Arg	Colon	[158]
440	CTT to CGT	Leu to Arg	Colon	[25]
445	CCT to CAT	Pro to His	Colon	[25]
450	GAC to GAG	Asp to Glu	Colon	[25]
450	GAC to CAC	Asp to His	Lung	[27]

inactivation in cancer. Although homozygous deletion of 18q21.1 is also a major mechanism of *Smad2* and/or *Smad4* inactivation, specific mutations of these genes results in defects in their functionality. Many of these mutations are found in the MH2 domain and can dysregulate Smad signaling by; (1) preventing phosphorylation, (2) weakening interactions during homo and hetero-oligomerization, and (3) altering interaction with other factors affecting transcription of Smad regulated genes [11, 49, 145, 146].

The mutations that map to the interface regions between the Smad4 monomers destabilize the trimeric complex. Mutations in the third loop on the face of the disk disrupt the formation of heteromeric complexes whereas mutations in the hydrophobic core destabilize the structure of the protein [48]. In a study assessing the role of the Smad2 P445H mutation, it was shown that upon over-expression of Smad2 P445H, the mutant protein could become phosphorylated by the RI receptor and associate with Smad3 or Smad4, but was unable to disassociate from the receptor. Moreover, when the mutant protein was phosphorylated upon ligand binding, Smad2P445H bound stably with wild type Smad2 and blocked the nuclear accumulation of Smad2 and subsequent Smad2 dependent transcription [147]. It was also shown that a missense mutation in a conserved amino acid in the MH1 domain of both Smad2 and Smad4 resulted in an enhanced autoinhibitory interaction between the MH1 and MH2 domains, which decreased protein stability [34]. Mutations in the MH1 domain of Smad4 were found to eliminate its ability to bind DNA even though they still retained the ability to form complexes with Smad3. Additionally, these mutant proteins exhibit decreased protein stability and an impaired ability to translocate to the nucleus [148]. Finally, a novel mechanism of inhibition of TGFβ signaling has been observed in mutations of a conserved residue of the MH1 domain of Smad2 and Smad4 which targets the mutant protein for rapid degradation *via* the ubiquitin-proteosome pathway [149].

The third *Smad* family member localized to 18q21, *Smad 7*, is an important regulator of Smad signaling pathways primarily by antagonizing TGFβ signaling *via* inhibition of R-Smads. Increased expression of Smad7 was shown in some pancreatic cancers, but there has been no evidence presented thus far for the existence of activating mutations or amplifications of the *Smad 7* gene [26,159]. The clustering of the *Smad* 2, 4 and 7 genes in a critical region of chromosome 18q21 which is deleted at high frequency in advanced tumors raises an intriguing possibility that there may be additional *Smad* genes localized to this region in addition to potentially unrelated tumor suppressor genes [11,24,142]. Similarly, *Smad3* and *Smad6* are located in the same general region on chromosome 15q21-22, which is frequently lost in a subset of breast, colorectal, lung and pancreatic tumors [160-162]. However, neither inactivating mutations, homozygous deletions, nor amplifications of *Smad3* or *Smad6* have been reported to date in human cancers [26,136,154,163-166].

Interestingly, the association of chromosome 18q loss [deletion of *Smad* gene(s)] with an advanced stage of human cancer and the observation of an increased malignant conversion frequency and decreased carcinoma latency in mice with disabled TGFβsignaling due to over-expression of the dominant negative TGFβ RII, illustrates that the inactivation of TGFβ signaling is a critical late event in the multi-step cancer progression [142,167-171]. Consistent with these studies are the recent findings that TGFβ signaling may have a role in metastasis as discussed in a subsequent section of this review.

GROWTH INHIBITION AND APOPTOSIS

TGFβcan inhibit cellular proliferation in a number of cell types. This inhibition occurs at the G_1 stage of the cell cycle and is accomplished through either one of the two following mechanisms: induction of cdk inhibitors such as p15, p21 and p27 or downregulation of Myc. $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$ are all members of the INK4 family of cyclin dependent kinase inhibitors that bind cdk4 and cdk6, to suppress their catalytic activity by preventing association with cyclin D [172]. TGFβ signaling mediated p15 induction and activation was originally shown in keratinocytes and was later implicated in stabilizing p15, enhancing the formation of p15-cdk4 complexes and inhibiting cyclinD-cdk4 complexes in mammary epithelial cells [173,174]. It has been recently reported that Smad2, Smad3, Smad4 and Sp1 are directly involved in p15 induction confirming previous reports that had implicated Sp1 and Smad3 in p15 induction [91,175,176]. Early in G_1, cyclinD and cdk4/6 come together to form complexes that sense mitogens. p15 binds to cdk4/6 and inactivates its catalytic activity, which prevents cyclinD from forming complexes with cdk4/6. By sequestering cdk4/6, the cells are prevented from progressing through G_1. Cyclin E-cdk2 and cyclin A-cdk2 complexes, respectively, regulate late G1 and early S phase. TGFβ can also block the cell cycle progression mediated by cyclin E-cdk2 and cyclin A-cdk2 complexes indirectly *via* p15 by disrupting these complexes. The Cip/Kip family of cdk inhibitors, which include $p21^{Cip/WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$, bind to these cyclin-cdk complexes causing their functional inactivation. CyclinD-cdk4/6 binds to p27 later in G_1 sequestering p27 and allowing cyclin E-cdk2 to become activated. TGF-β mediated induction of p15 allows its binding to the cyclin D-cdk4/6 complexes which frees p27, so that it can bind to cyclin E-cdk2 and prevent progression into S phase [174, 177, 178].

p21 is also known to bind to G_1 cdks, and recent reports suggest that p21 could also play an important effector role in TGFβ growth suppression. While Smad3, Smad4 and Sp1 cooperate to activate the p21 promoter in HepG2 cells in response to TGFβ, the DNA binding domain mutants of Smad3 and Smad4 as well as overexpression of Smad7 inhibited the induction of p21 by TGFβ [92]. Despite general acceptance that p21 induces cell cycle arrest, clues to the molecular basis of its function have only recently started to emerge. HPV16 immortalized human ectocervical cells treated with TGFβ were shown to have increased p53 levels that correlated with time and dose. In these cells, p21, bax and Mdm2 levels exhibited a concurrent increase with similar kinetics, while no distinguishable changes in the protein levels of cyclin D, cyclin E, cdk4, cdk6, cdk2, p27, p16 or RNA levels of p15 were observed. However, the catalytic activity of cdk2 was decreased, possibly by binding

to p21, which was shown to coimmunoprecipitate with cdk2 upon TGFβ stimulation [179]. In gastric carcinoma cells, it has been reported that p21 inhibited the kinase activity of cyclin D and cyclin E associated cdks resulting in hypophosphorylation of p130, a member of the RB family of proteins that regulate E2F [180]. Additionally, adenoviral E1A, a potent stimulator of cellular proliferation has also been shown to block TGFβ induced induction of p15 and p21. The decrease in p15 appears to be at the level of transcriptional regulation and is dependent upon E1A's ability to bind p300 but independent of E1A's pRb binding activity [181]. Despite all these experimental data suggesting that TGFβ growth inhition is mediated *via* cdk inhibitors, it is also important to note that the growth inhibitory effects of TGFβ are not solely mediated by cdk inhibitors as p15-/- and p27-/- mouse embryo fibroblasts are also sensitive to TGFβ mediated growth inhibition [182, 183].

Downregulation of cdk activators is another mechanism by which TGFβ can exert its growth suppressive effects. The cdc25A phosphatase removes tyrosine phosphorylation of cdk4 and cdk6, which in turn, activates these kinases. TGFβ downregulates cdc25A, which prevents the removal of these inhibitory phosphate groups from and inhibits the catalytic activity of cdk4 and cdk6 [184]. The cdc25A downregulation involves a transcriptional repressor complex containing E2F, p130, and HDAC1, which bind to the promoter of cdc25A [1]. However, two studies have demonstrated that this downregulation of cdc25 is subsequent to p21 induction, which occurs very rapidly (< 1 hr) while the induction of p15 occurs at a later time point [184, 185].

Growth suppression by TGFβ may also be activated upstream of cdk inhibitors by downregulating c-Myc. c-Myc is a ubiquitous promoter of cell growth and can act as both a transcriptional activator or repressor dependent upon the cofactors with which it associates [186]. The downregulation of c-Myc occurs very rapidly in many cells that are sensitive to TGFβ growth inhibition [187]. The downregulation of c-Myc in response to TGFβ has been proposed to be an essential early event prior to the induction of p15 and p21, as overexpression of c-Myc blocks p15 and p21 induction and ablates the cell cycle arrest [188,189]. A positive association between c-Myc and cdc25A has been demonstrated and opens the possibility of another level of regulation of cdc25A by TGFβ [190]. A genome wide analysis of rapid TGFβ gene responses comparing human mammary epithelial cells with breast cancer cells demonstrated that c-Myc repression was selectively lost in the breast cancer cells. TGFβ induces a Smad complex that binds to a TGFβ inhibitory element in the c-Myc promoter. In the breast cancer cell line, these complexes were defective and the authors suggest that this Smad complex is a target of oncogenic signals in breast cancer [191]. Dual roles for the Myc-interacting zinc-finger protein 1 (Miz1) in response to TGFβ signaling have recently been discovered, providing clues to the molecular basis of some of these end effects. Miz-1 relieves repression of $p15^{INK4b}$ expression by interacting with Myc, preventing its recruitment to its promoter, and by enabling Smad protein complex mediated transactivation. [192].

While TGFβ has also been shown to induce apoptosis in a number of cell types (hepatocytes, myeloid cells and epithelial cells), organs and tissues, the mechanisms by which apoptosis is induced by TGF-β are poorly understood. TGFβ induced apoptosis is essential for normal development of the neural crest, interdigital fields of the limb and the mammary gland ductal system [193-195]. There is also mounting indirect evidence implicating TGFβ mediated apoptosis in the elimination of preneoplastic cells and that the abrogation of this pathway results in an increased rate of carcinogenesis and/or metastasis. The mechanisms regulating TGFβ mediated apoptosis are just coming to light. There has been an increasing body of literature implicating Smad7 in the pathway. Smad7 expression is increased in rat prostatic epithelial cells undergoing apoptosis due to castration, and TGFβ induced apoptosis was ablated by inhibiting Smad7 levels in a number of cell lines [196]. Smad7 expression increased apoptosis in Mv1Lu cells in response to TGFβ and serum withdrawal. Furthermore, Smad7 decreased the activity of NF-κB, a potent survival factor, and sensitized cells to various forms of cell death [197]. Smad3 and AP-1 may also play a role in this pathway as overexpression of Smad7 or dominant negative Smad3 was shown to block TGFβ induced apoptosis [198]. Furthermore, the JunD-FosB-AP1 complex is also activated in TGFβ dependent apoptosis. Dominant negative FosB inhibited apoptosis but not growth suppression in these cells [198]. These observations would predict that directly or indirectly disabling Smad3, Smad7 or other signaling molecules of the TGFβ signaling pathway involved in apoptosis could facilitate tumor formation. Although gastrointestinal tumors have been observed in Smad3 knockout mice, so far there has been no evidence for mutational inactivation in human tumors suggesting that there might be indirect mechanisms for inactivation of this molecule in cancer.

Bcl and caspase family members have also been implicated in mediating the apoptotic pathway induced by TGFβ[7,199]. The NRP-154 rat prostate epithelial cell line can be induced to undergo apoptosis in response to TGFβ. In this model, TGFβ downregulates bcl-xL and PARP expression, promotes cytochrome c release and upregulates expression of caspases 3 and 9. Overexpression of bcl-xL prevented apoptosis by blocking cytochrome c release, activation of caspases 3 and 9 and cleavage of PARP. Interestingly, these cells were still sensitive to TGFβ cell cycle arrest suggesting that there may be independent signaling events mediating these two processes [200]. A recent report suggests that bcl-xL downregulation is a result of reactive oxygen species (ROS) production by TGFβ mediated effects. Decreased levels of bcl-xL cause a decrease in mitochondrial transmembrane potential, which may lead to the release of cytochrome c and the activation of caspase 3, resulting in apoptotic cell death [201]. Overall, these observations strongly imply that cell specific growth inhibitory or apoptotic responses mediated by TGFβ *via* the Smads may become disabled in tumor cells enabling the uninhibited growth of tumors.

SMADS CROSSTALK WITH OTHER SIGNALING PATHWAYS

It is becoming increasingly clear that the TGFβ signaling pathways are part of a signaling network that can lead to

numerous biological end effects. Signaling cascades involving Ras, JNKs, p38, and JAKs have been shown to interact with TGFβ signaling at various levels leading to both synergistic and suppressive end results dependent upon the cellular context. MAP kinase pathways lead to the activation of either the ERK1/2, JNK or p38 kinase. The MAP kinase pathway is frequently dysregulated in cancer, most often due to oncogenic mutations in Ras, which has been shown to be an early event in a number of cancers [202-204]. Ras signaling has also been shown to be both antagonistic as well as cooperative with TGFβ signaling. Ras antagonism can occur at various levels. Ras stimulates the activation of cyclin dependent kinases (CDKs) which are important in cellular progression through the cell cycle and hence proliferation. TGFβ signaling induces the production of CDK inhibitors that can cancel the effects of CDK activation by Ras [177,205]. It has also been proposed that activation of the MAP kinase cascade by Ras can inactivate Smad signaling by sequestering Smad2 and Smad3 in the cytoplasm to such an extent that even overexpression of Smad4 is not able to restore TGFβ signaling [206]. Additionally, oncogenic Ras signaling has been shown to increase the stability of the Smad co-repressor TGIF due to phosphorylation resulting in the suppression of expression of CDK inhibitors such as p15 [207]. Ras can also cooperate with TGFβ signaling, and there is a growing body of evidence suggesting that Ras may "reroute" TGFβ signaling in epithelial cells. Oncogenic Ras in mammary epithelial cells allows these cells to transdifferentiate into a highly invasive and metastatic phenotype while inhibiting Smad dependent growth inhibition [208]. Additionally, breast cancer cells with a constitutively activated Ras have an increased ability to metastasize to the bone in response to TGFβ [209].

Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) have also been reported to activate Smad dependent gene transcription *via* an ERK dependent pathway in epithelial cells [210]. However, when phosphorylation of the linker region of Smad2 was catalyzed by MEKK1, it prevented the nuclear localization of Smad2 and thus Smad dependent transactivation [211]. Since phosphorylation of Smad2 usually occurs in the C-terminus of the protein in response to TGFβ, it has been postulated that the sites where R-Smads are phosphorylated would ultimately determine whether growth inhibition or growth stimulation would occur during the signaling cascades.

TGFβ can itself activate numerous MAP kinase pathways, including the MKK4-JNK and MMK3-p38 pathways that can lead to both Smad-dependent and Smad-independent transcription [85,212-215]. The activation of these cascades by TGFβ may involve Rho-like guanosine triphosphatases [211]. TGFβ activated kinase 1 (TAK1) may also be involved in TGFβ signaling through p38 [216-218]. While TGFβ activates the hematopoetic progenitor kinase (HPK) and an association between HPK and TAK1 has been shown, a direct link between TAK and TGFβ RI is still unresolved [218]. TGFβ activation of JNK or p38 can mediate transcription by activating AP-1 complexes *via* phosphorylation of c-Jun or CRE-regulatory complexes through ATF2 phosphorylation [85,213]. The specificity of these end effects may be mediated by the specific interactions between Smads and ATF2 or Jun complexes that have recently been reported [85,87,216,219]. Interestingly, JNK has also been shown to phosphorylate Smads at undetermined sites within the linker region [220].

Finally, there is also emerging evidence to support crosstalk between TGFβ signaling and the JAK/STAT andNF−κB signaling pathways. Interferon-γactivates JAK tyrosine kinases which in turn activate STAT proteins. It has now been reported that Interferon-γ inhibits TGFβ signaling by direct STAT-mediated transactivation of Smad7 [221]. Smad7 activation and inhibition of TGFβ signaling has also been shown to be mediated by NF−κB/RelA [222]. While all the details regarding the exact interactions that occur between TGFβ signaling molecules and members of other signaling cascades remained unresolved, it is becoming increasingly clear that TGFβ signaling is very complex with cell type and cellular micro- and macro-environment dictating which biological end results will occur.

TGFβ SIGNALING IN ANGIOGENESIS AND METASTASIS

During early stages of tumor development, TGFβ functions to suppress cell-cycle progression and inhibit tumor growth [173,177,223]. However, in later stages, human tumor cells generally develop resistance to TGFβ-mediated growth inhibition [224]. In advanced cancers, TGFβ ceases to function in tumor suppression and adopts the converse role of enhancing metastatic spread. Dissemination of malignant cells from a primary tumor to distal sites in the body is the principal cause of death in cancer patients. Metastasis can occur through a variety of mechanisms, including direct invasion of surrounding tissue (*per continuitatem*), dissemination *via* the blood vasculature (hematogenous metastasis), and/or through the lymphatic system (lymphatic metastasis) [225]. Overexpression of TGFβ is detected in several advanced and metastatic human tumor types, including prostate, mammary, and renal cell cancers [226-229]. Elevated levels of TGFβ are usually detected in the microenvironment surrounding the tumor and in the tumor stroma [230]. The excess TGFβ may enhance tumor progression by promoting local tissue invasion and by inducing tumor angiogenesis. TGFβ appears to play a role in both *per continuitatem* and hematogenous metastatic spread.

Metastasis *via* local tissue invasion is apparently promoted by TGFβ signal transduction by altering the properties of the cell [208,223,231]. TGFβ stimulates the conversion of fully polarized, non-invasive epithelial cells to an invasive mesenchymal, spindle cell-phenotype [231]. The epithelial to mesenchymal transition (EMT) involves decreased expression of genes important in cell-cell adhesion and increased expression of genes involved in cell-extracellular matrix connections [232]. The resulting cells display enhanced migration and invasion into surrounding tissues and demonstrate autocrine production of TGFβ, which is essential to sustain the invasive property of the tumor cell [231]. On the contrary, interference with TGFβ receptor signaling converts invasive, metastatic, mesenchymal cells to non-invasive cells with an epithelial phenotype [231]. Therefore, the TGFβ receptors may be

promising targets for late-stage cancer therapeutics to revert invasive cells to a more benign phenotype.

Tumor angiogenesis, that is, the onset of neovascularization within a primary tumor, is essential for both tumor expansion and metastasis. The newfound access of the tumor to the host bloodstream not only affords necessary oxygen and nutrients to tumor cells, but also provides a route through which these cells may disseminate to distal sites [233]. The role of TGFβ in blood vessel formation remains a highly complex process. TGFβ exerts a biphasic effect on angiogenesis induced by vascular endothelial growth factor (VEGF). *In vitro*, high concentrations of TGFβ inhibit endothelial invasion and capillary lumen formation, whereas lower concentrations of TGFβ synergistically function to increase endothelial cell invasion mediated by VEGF or basic fibroblast growth factor (bFGF) [234, 235]. However, *in vivo*, TGFβ has been shown to function in stimulating angiogenesis [236]. TGFβ-1 knockout mice were found to suffer from defective vasculogenesis and embryonic lethality, supporting an essential role for TGFβ signaling in blood vessel formation [237].

The activin receptor-like kinases (ALKs) are TGFβ RI receptors, which seem to play a role in blood vessel formation and remodeling. ALK1 and ALK5 induce different TGFβ signaling pathways that determine the phenotype of the endothelium during angiogenesis; endothelial cells alternate between an activation phase and a resolution phase during blood vessel formation [238]. TGFβ signaling through ALK5 seems to promote passage into the activation phase, which is characterized by migration and proliferation of endothelial cells to generate vessel formation. However, signaling through ALK1 appears to induce the resolution phase in which endothelial cells cease migration and proliferation and the basement membrane becomes reconstituted [239]. It has been speculated that ALK5 has a higher sensitivity to TGFβ than ALK1; low concentrations of TGFβ would therefore only activate ALK5 and not ALK1, thus favoring endothelial cell proliferation. Higher concentrations of TGFβ would bind and activate ALK1, which inhibits the ALK5 pathway and thus concludes the process of angiogenesis [239]. As demonstrated in the aforementioned *in vitro* studies, varying concentrations of TGFβ apparently affect angiogenic activity *via* the mediation of VEGF and bFGF; it can be hypothesized that TGFβ signaling through ALK1 and ALK5 regulates angiogenesis in a similar manner. However, further studies are required to elucidate the exact nature of the signaling events mediated by ALK1 and ALK5.

The recruitment of excess TGFβ to areas of angiogenic activity may be regulated by CD105 (endoglin). This glycoprotein is similar to the RIII receptor for TGFβ, and is expressed predominantly in proliferating endothelial cells. Expression levels of CD105 are particularly elevated in endothelial cells of tumor blood vessels, but are virtually undetectable in blood vessels of normal tissue [240-242]. Overexpression of CD105 has been linked to blood vessel formation, whereas CD105 null mice are severely defective in angiogenesis and die *in utero* [243]. Thus, expression of CD105 may mediate the cellular proliferation effects of TGFβ in the blood vessel endothelium, promoting angiogenesis and subsequently metastasis.

Due to the limited availability of data, the overall role of TGFβ signaling in stimulating angiogenesis appears highly complex and is largely unresolved. However, a recent report demonstrates that Smad4 inhibits expression of VEGF, a potent inducer of angiogenesis, and increases expression of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis [244]. Hence, inactivation of Smad4, as observed at high frequency in colorectal and pancreatic cancers, would implicate promotion of tumor angiogenesis and metastasis. This notion has been further supported by recent experimental data from the analysis of human colorectal cancer where a strong correlation between higher frequency of Smad4 gene mutations and distant metastases relative to non-metastatic forms of colon cancer was observed [154]. This data is also consistent with a role for Smad4 inactivation in the stimulation of angiogenesis, which could in turn permit hematogenous metastasis. However, it seems highly likely that the function of TGFβ in tumor blood vessel formation is reliant upon both the tumor type and the particular mutation in the TGFβ signaling pathway.

TGFβ is also known to induce the expression of various components of the extracellular matrix (ECM), including collagen, fibronectin, tenascin, vitronectin, proteoglycans and integrins. In some cell types, this growth factor also functions to inhibit degradation of the matrix by repressing expression of proteases, including plasminogen activators, collagenase and stromelysin and through activating protease inhibitors, such as TIMP1 and PAI-1 [245-248]. Degradation and remodeling of the ECM are key events in both angiogenesis and metastasis. In order to disseminate, tumor cells must detach from neighboring cells and escape from the tissue of origin *via* either hematogenous or lymphatic routes and hence the breakdown of the ECM would hasten the flight of the tumor cells to distal sites [249]. Thus, blood vessel and possibly lymphatic vessel formation is facilitated by degradation of the ECM. The breakdown of connective tissue barriers creates a path through which endothelial cells are able to migrate, adhere and proliferate, generating new vessels which may grow toward and infiltrate the tumor mass.

Despite a plethora of evidence presented in the literature for promotion of ECM formation by TGFβ, contradictory roles for TGFβ in the maintenance of the ECM have also been observed in the tumorigenic process. In malignant cells that have acquired TGFβ-resistance, one may expect that pathways that induce expression of ECM molecules would not be intact. However, TGFβ retains its ability to induce ECM as well as to positively stimulate breakdown of the ECM through induction of type IV collagenases. These matrix metalloproteases are involved in the degradation of basement membrane and promote metastatic characteristics, which are often observed in studies using tumor cell lines as the experimental system [250-253]. The role of TGFβ in ECM maintenance is primarily mediated through RI receptor signaling [254]. However, it has been postulated that TGFβ regulates certain ECM molecules independently, possibly through different receptor subtypes or alternative signaling molecules [248]. In contrast to the degradation of ECM promoted by TGFβ during tumor metastasis, other observations show an inhibition of ECM degradation by TGFβ-mediated stimulation of the synthesis of PAI-1 in both

primary and metastatic tumors [248]. Therefore, the role of TGFβ in promoting ECM degradation, and thus allowing metastasis, is not globally applicable to all cancers and may be dependent on the specific mutated targets in the TGFβ signaling pathways.

Recent studies have provided additional evidence to confirm that TGFβ-mediated growth suppression and apoptosis may operate independently of the angiogenic and metastatic processes. Activated Ha-Ras collaborates with TGFβ to induce the phenotypic invasiveness of mammary epithelial tumor cells, whereas Raf-induced TGFβ production blocks its apoptotic but not its invasive responses in epithelial cells [231, 255]. Additionally, work using breast cancer cell lines has shown a requirement for Smad2 and Smad4 in inducing trans-differentiation in cells with low levels of RI receptors [256]. Furthermore, a recent study reports that blocking TGF-β signaling with truncated RII receptors has no effect on the local growth of the tumor, but significantly decreases metastasis to the bone [209].

Although there is increasing evidence for TGFβ involvement in metastasis through both local tissue invasion and hematogenous spread, it remains to be determined whether TGFβ plays a role in lymphatic metastasis. Tumor cell dissemination *via* the lymphatic system has received secondary consideration relative to tumor metastasis *via* local tissue invasion or *via* the blood vascular system. With the recent cloning of the lymphatic vessel specific growth factors, VEGF-C and VEGF-D, focus has shifted toward the lymphatic system as an important medium for metastasis [257-259]. These observations prompt one to ask whether a relationship exists between TGFβ signaling and lymphatic metastasis, or more specifically, between TGFβ and VEGF-C and/or VEGF-D. A refined understanding of the molecular mechanisms underlying metastasis will elucidate the contribution of TGFβ signaling to tumor cell dissemination.

TGFβ AND IMMUNE SUPPRESSION

The critical roles of TGFβ in regulating the immune system have recently received increasing attention. TGFβ is a multi-functional cytokine with a number of roles in the immune system [4, 260]. Many immune cells, including lymphocytes, macrophages and dendritic cells, produce TGFβ; this expression is regulated by both autocrine and paracrine pathways, and TGFβ mediates the differentiation, selection, apoptosis, activation and proliferation of these immune cells [4, 261]. The characterization of the Smad family of proteins has helped to elucidate the molecular mechanisms underlying the effects of TGFβ in the immune system, including cross talk with other cytokine and lymphokine pathways [221, 262].

T lymphocytes are regulated by TGFβ at all stages of development, from differentiation to activation and proliferation [263,264]. Some subsets of activated T cells (Th3 cells) are able to synthesize and secrete TGFβ, which typically inhibits production of interkeukin-2 (IL-2), thus suppressing T cell proliferation. Consistent with these observations, TGFβ1 null mice exhibit an increased expression of IL-2, class II MHC antigen and primary expansion of $CD4^+$ T cells [265, 266]. Such mice also develop a multifocal inflammatory disease with autoimmune manifestations, including production of autoantibodies [267]. During T cell education, TGFβ may regulate maturation of double positive T cells from $CD4^-CD8^{low}$ precursors; in the absence of TGFβ1, double positive thymocytes are generated too rapidly to allow appropriate selection processes to transpire. Dysregulated production of $CD4^+CD8^+$ T cells in these mice may be exacerbated by defects in apoptosis of T cell subsets, thus causing autoimmune disease [263].

Cytotoxic T cells (CTLs), or class I MHC- restricted T cells, can provide an effective anti-tumor defense in cell-mediated immune responses. These effector cells are responsible for direct killing of virus-infected or allogeneic cells [268]. CTLs exert their effect by lysing cells through one of two mechanisms: the perforin/granzyme B pathway or the Fas/Fas ligand pathway. TGFβ1 plays a role in determining cytotoxicity by regulating the repertoire of gene expression in the CTLs. Several studies have demonstrated *in vitro* that TGFβ1 inhibits perforin mRNA expression and thus suppresses the perforin/granzyme B cytotoxic pathway; however, TGFβ1 has no effect on the Fas/FasL cytotoxic pathways [269, 270]. TGFβ1 may also decrease immunosurveillance by CTLs in a more indirect manner through suppressing expression of helper T cells. CD4+ helper T cells (Th cells), or class II MHC restricted T cells, are not generally cytotoxic to tumors; however, these cells may play a role in the anti-tumor response by providing cytokines for the effective development of CTLs [268]. Recent studies have shown that TGFβ1 suppresses memory Th1 T cell maturation by down-regulating expression of the β2 chain of the IL-12 receptor; IL-12 is required for a Th1 cell to develop from a naïve CD4+ T cell. TGFβ1 also inhibits the development of Th2 cells. During cancer progression, TGFβ1 suppression of the expression of perforin and prevention of the maturation of Th1 and Th2 cells may thus inhibit anti-tumor immune responses.

It remains difficult to develop a comprehensive view of the effect of TGFβ on T lymphocytes as TGFβ works not only directly but also indirectly on T cells by affecting antigen-presenting cells (APCs). Dendritic cells (DCs) are typical APCs in both lymphoid and non-lymphoid organs; they function to present peptide antigens to helper T cells and to initiate antigen-specific T cell proliferation. Several studies have reported that TGFβ inhibits *in vitro* activation and maturation of lymphoid tissue type DCs [271]. TGFβ may inhibit regulation of T cell costimulatory molecules on the surface of these DCs and thus reduce their antigen presenting capacity. The immunosuppressive role of TGFβ in cellular immunology is therefore due to its inhibitory effect on both effector T cells and APCs.

TGFβ may also mediate several aspects of the maturation and differentiation functions of B cells. *In vitro,* TGFβ can inhibit B cell proliferation, antibody secretion and expression of surface molecules, including antigen receptors. In humans, TGFβ is also involved in directing switch recombination in immunoglobulin isotype IgA; in TGFβ null mice, high levels of autoantibodies, predominantly IgG, are detected [272, 273]. Several human and murine B lymphoid malignancies have been identified in which B cells become

resistant to the growth inhibitory effects of TGFβ; these malignant B cells express substantial amounts of active TGFβ. These insensitivities may be derived from defects in any of the players of the TGFβ signaling pathway, including the Smads. Further studies are necessary to determine the molecular basis of these defects.

Studies of pathogens suggest that increased production of TGFβ may lead to decreased immune surveillance. The immune response to a variety of pathogens, including viruses, bacteria, yeast and protozoa, is regulated by TGFβ. *Trypanosoma cruzi,* a protozoan parasite, infects macrophages and suppresses their antibacterial activity; these macrophages are stimulated by the parasite to produce increased levels of TGFβ [274]. Studies, which have focused on mycobacteria and viruses, including the human immunodeficiency virus (HIV), have reported that TGFβ has both a negative influence on host immune response and a beneficial effect on the survival and growth of intracellular pathogens [275].

TGFβ may allow tumor progression through its ability to suppress immune surveillance. An increase in the expression of TGFβ is common in carcinomas resistant to TGFβ-mediated growth inhibition and is also apparently important for tumor progression; this association may also play a role in suppression of immune surveillance [276-278]. Expression of TGFβ allows disseminating tumor cells to permeate the body without being attacked by mediators of the immune response such as cytotoxic T cells and macrophages. In some primary tumor cells, increased expression of TGFβ and interleukin 10 (IL-10), another potent immunosuppressive factor, has been detected; expression of such genes may explain how tumors suppress CTL function and escape from the immune response [279, 280]. The analysis of genes expressed by tumor cells using high throughput methods may provide the critical data required to discover which other cytokines or factors are involved in the effective immune suppression achieved by tumors [281-283].

GENE KNOCKOUT STUDIES

TGFβ expression begins early in development, and gene targeting approaches have been used to elucidate the *in vivo* functions of both TGFβ isoforms and Smads. TGFβ1 (-/-) mice develop a progressive wasting syndrome resulting in death within a few days [254, 255]. Detailed analysis showed infiltration of lymphocytes and macrophages and necrosis in many of the organs, especially the heart and lungs [254-257]. Many of the lesions found in these mice resembled those found in autoimmune disorders, suggesting a role for TGFβ1 in immune cell proliferation as we have already described in detail in the previous section of this review [235, 258]. An embryonic phenotype has also been observed in the TGFβ1 (-/-) mice as only 50% of the conceptuses reach parturition [207, 254, 255]. These mice die by E10.5 due to defective yolk vasculogenesis as well as defective hematopoesis [207]. However, the TGFβ 1(-/-) mice, when derived from a 129 background, develop nonmetastatic colon cancer if they are rescued from autoimmune disease [259, 260]. Additionally, TGFβ1 may also play a role in susceptibility to carcinogenesis. TGFβ1 (+/-) mice have an enhanced propensity to develop lung and liver cancer when treated with carcinogens.

TGFβ2 (-/-) mice die from congenital cyanosis shortly before or during birth. They have profound craniofacial, eye, spinal column, cardiac, lung, limb and urogenital defects [261]. TGFβ3 (-/-) mice die within 24 hrs of birth. They have cleft palates and abnormal pulmonary histology, but most of the remaining organs are normal [262, 263].

TGFβ receptor RII knockouts result in embryonic lethality. Null mice die at E10.5 due to defects in yolk sac hematopoesis and vasculogenesis [264]. These mice resemble the TGFβ1 (-/-) mice suggesting that the TGFβ-receptor RII may play a role in hematopoesis and endothelial differentiation.

Smad knockout studies have revealed that all of the Smad knockouts exhibit developmental defects (Table 5). While the Smad 2, 4 and 5 knockouts are embryonic lethal, the Smad 3 and 6 knockout mice survive to term. Smad 2 (-/-) mice die before E8.5 [265-268]. These Smad2 null embryos do not form a head fold or primitive streak, lack the extraembryonic portion of the egg cylinder, have no mesoderm formation and do not undergo gastrulation. Smad 3 null mice are born viable and fertile [269, 270]. However, these mice die between one and eight months of age due to

Table 5. Smad Knockout Mice

Gene	-/- Phenotype	References
Smad2	Embryonic lethality by E7.5-E8.5. Defects in egg cylinder elongation, mesoderm formation, and gastrulation. Anterior-posterior axis formation abnormalities.	[295-298]
Smad3	Death within 1-10 months. Metastatic colorectal cancer at 4-6 months of age. Immune dysregulation, severe mucosal infection, accelerated wound healing, osteoporosis and skeletal defects.	[299-301]
Smad4	Embryonic lethality by E7.5-E8.5. Growth retardation. Abnormal visceral endoderm formation, absence of mesoderm and lack of gastrulation. Primary defects in extraembryonic tissue development and abnormalities in anterior-posterior axis formation.	[302, 303, 304]
Smad5	Embryonic lethality by E9.5-E10.5. Defects in angiogenesis, mesenchymal apoptosis and in gut, heart and craniofacial development.	[306-308]
Smad6	Cardiovascular abnormalities and defects in endocardial cushion transformation.	[309]

chronic infection, suggesting that Smad 3 is a player in immune responses [269, 271]. Fibroblasts from the Smad 3 null mice have been shown to be partially resistant to TGFβ dependent growth inhibition [269]. In some genetic backgrounds, Smad3 (-/-) homozygotes develop colorectal tumors ranging from hyperplastic lesions and polyps to highly aggressive tumors, which are very invasive and highly metastatic, with dissemination to the lymph nodes [270]. The Smad 4 null phenotype is similar to that of the Smad 2 knockout. Smad 4 (-/-) mice die before E7.5, are severely growth retarded, fail to gastrulate, fail to form the egg cylinder, and show abnormal visceral endoderm development [272]. It was originally reported that Smad 4 heterozygous mice show no increase in tumorigenicity [272]. However, a recent report has shown that Smad4 heterozyous mice develop gastric polyps that can develop into tumors at a late age [273]. Furthermore, a compound heterozygote mouse carrying mutations in the the *APC* (adenomatous polyposis coli) gene (delta716) and Smad4 exhibited intestinal polyps which developed into malignant tumors to a greater extent than those in the simple *APC* heterozygotes, suggesting that mutations in Smad4 play a significant role in the malignant progression of colorectal tumors [274]. Smad 5 (-/-) mice die at E9.5 – E11.5, and have numerous defects in angiogenesis, including enlarged vessels and fewer smooth muscle cells. Smad5 (-/-) homozygotes have left/right asymmetry, craniofacial abnormalities, and they undergo extensive mesenchymal apoptosis [275-277]. Smad 6 knockouts are viable, but have a number of cardiac abnormalities, suggesting a role for Smad 6 in the development and homeostasis of the cardiac system. These Smad 6 (-/-) mice have hyperplasia of the cardiac valves, outflow tract septation defects, aortic ossification and elevated blood pressure [278].

FUTURE PERSPECTIVES

The TGFβ signaling pathways have gained importance in cancer research due to their opposing roles in both tumor suppression and metastatic cancer promotion elicited *via* specific end effects. Many researchers have reported conflicting evidence when undertaking very similar experiments. How can some groups detect growth inhibitory effects, and hence resistance to tumorigenesis, while others report invasiveness and metastasis? Often times, cell type specificity may be explained by a unique set of constitutively expressed and/or inducible protein factors which affects the internal cellular environment. However, in the case of TGFβ, the answer is much more complex due to the influence of the local and/or overall environment of the target tissue. TGFβ signaling can be affected by externally introduced agents, as demonstrated in studies showing that smoking causes a further increase in the plasma levels of TGFβ in patients with diabetes, and that exposure to hydrogen peroxide causes the induction of TGFβ1 and an associated increase in ECM components [310, 311]. The identity and roles of the various chemicals or other agents introduced from the environment, which affect TGFβ signaling and hence influence the development or metastatic spread of tumors, is still awaiting further research. These exogenous factors may cause specific genetic or epigenetic alterations at certain hot spots ultimately leading to localized deletions such as that of chromosome 18q and thus inactivation of target genes [11,23,24,142,312,313]. Alternatively, these factors may cause non-genetic altera-tions of the functional outcomes of the various signaling events, such as the rerouting of standard signaling pathways. The end results of these changes that may contribute to cancer could be comprised of, but are not limited to, the prevention of cellular turnover due to ablation of normal TGFβ growth inhibition, inhibition of apoptosis, increase in malignant conversion of cells affording angiogenesis, tumor invasion and metastasis and enhancement of the events that normally disable immunosuppressive effects.

Although the molecular basis of TGFβ signaling events are becoming increasingly clear due to the discovery of critical signaling mediators such as the Smad proteins, much work remains for the research community. Of importance is the need to unravel the end effects resulting from the various combinatorial signaling events at the level of each signaling event, the signaling cross talk and the environmental effects that may ultimately be responsible for the development of cancer. Additionally, there is an emerging general acceptance that human TGFβ signaling is mediated by Smads, involving different R-Smads and the only known Co-Smad, Smad4. However, recent studies might suggest otherwise due to the evidence for TGFβ mediated growth inhibition in Smad4 disabled cells and due to the demonstration of the existence of Smad4 homologues in other vertebrates [46, 314]. These observations strongly suggest that there might be other TGFβ signaling pathways operative in the cells, which involve other Smad4 homologues or Smad4 independent events. The rapid development of high throughput genomic methodologies and data produced from the application of these methods is expected to aid in understanding these complexities and in providing new leads for delineating TGFβ–mediated signaling events. The availability of human sequence data can be taken advantage of to speed up the discovery of novel genes as well as the discovery of new members of the *Smad*, *TGFβ* ligand and *TGFβ* receptor families [315, 316]. Furthermore, the systematic use of high throughput methods such as gene expression microarrays, SAGE (serial analysis of gene expression) and proteomics in conjunction with gene knockouts in cell lines and animal models may ultimately help in determining the various players in the signaling events as well as in eliciting their biological end effects [281-283]. These studies would provide an overall picture of TGFβ signaling and help to determine the critical steps or target genes in which inactivations may lead to cancer, thus aiding in the identification of nodal points for targeting therapeutic applications to contain and combat cancer.

ACKNOWLEDGEMENTS

Work in the authors' laboratory is supported by grants from the American Lung Association (RG-013-N), American Cancer Society (72-001-26-IRG), NIH (RO1 ES10377), Evans Medical Foundation, Dolphin Trust Investigator Award from the Medical Foundation and Career Development Award from the Department of Defense (DAMD 17-01-1-0160). R. L. F, H. J. L, K-h. C and J. F. P

are supported by the ALA, Medical Foundation and NIH training grant (T32HL07035).

REFERENCES

[1] Massague, J., Wotton, D. (2000) Transcriptional control by the TGFβ/Smad signaling system. *EMBO J.,* **19**(8), 1745-54.

[2] De Caestecker, M.P., Piek, E., Roberts, A.B. (2000) Role of transforming growth factor-beta signaling in cancer. *J. Natl. Cancer Inst.,* **92**(17), 1388-402.

** TGFβ signaling and the various points at which inactivation in cancer could occur is dicussed.

[3] Massague, J., Blain, S.W., Lo, R.S. (2000) TGFβ signaling in growth control, cancer, and heritable disorders. *Cell,* **103**(2), 295-309.

** A brief account of the defects in TGFβ signaling that could lead to heritable disorders and cancers is described.

[4] Letterio, J.J., Roberts, A.B. (1998) Regulation of immune responses by TGFβ. *Annu. Rev. Immunol.,* **16,** 137-61.

[5] Massague, J. (1996) TGFβ signaling: receptors, transducers, and Mad proteins. *Cell,* **85**(7), 947-50.

[6] Ohta, S., Yanagihara, K., Nagata, K. (1997) Mechanism of apoptotic cell death of human gastric carcinoma cells mediated by transforming growth factor beta. *Biochem. J.,* **324** (Pt 3), 777-82.

[7] Saltzman, A., Munro, R., Searfoss, G., Franks, C., Jaye, M, (1998) Ivashchenko Y. Transforming growth factor-beta-mediated apoptosis in the Ramos B-lymphoma cell line is accompanied by caspase activation and Bcl-XL downregulation. *Exp. Cell Res.,* **242**, 244–254.

[8] Hojo, M., Morimoto, T., Maluccio, M., Asano, T., Morimoto, K., Lagman, M., Shimbo, T., Suthanthiran, M. (1999) Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature,* **397**(6719), 530-4.

[9] Gold, L.I. (1999) The role for transforming growth factor-beta (TGFβ) in human cancer. *Crit. Rev. Oncog.,* **10**(4), 303-60.

[10] Markowitz, S. (2000) TGFβ receptors and DNA repair genes, coupled targets in a pathway of human colon carcinogenesis. *Biochim. Biophys. Acta,* **1470**(1), M13-20.

** A detailed account of the TGFβ type II receptor inactivations in colon cancer is described.

[11] Thiagalingam, S. (2001) Molecular detection of Smad4/Smad2 alterations in colorectal tumors: Colorectal Cancer Methods and Protocols. In Methods in Molecular Medicine. *S. M. Powell (Ed) Humana Press Inc., New Jersey,* **50,** 149-165.

** This chapter outlines molecular genetic analytical methods to evaluate the two most commonly mutated Smad genes, *Smad2* and *Smad4*.

[12] Massague, J. (1998) TGFβ signal transduction. *Annu. Rev. Biochem.,* **67,** 753-791.

[13] Roberts, A.B. (1998) Molecular and cell biology of TGFβ. *Miner. Electrolyte Metab.,* **24**(2-3), 111-119.

[14] Sporn, M.B., Roberts, A.B. (1988) Peptide growth factors are multifunctional. *Nature,* **332,** 217-219.

[15] Greenwald, J., Fischer, W.H., Vale, W.W., (1999) Choe, S. Three-finger toxin fold for the extracellular ligand-binding domain of the type II activin receptor serine kinase. *Nat. Struct. Biol.,* **6**(1), 18-22.

[16] Kirsch, T., Sebald, W., Dreyer, M.K. (2000) Crystal structure of the BMP-2-BRIA ectodomain complex. *Nat. Struct. Biol.,* **7**(6), 492-6.

[17] Huse, M., Chen, Y.G., Massague, J., Kuriyan, J. (1999) Crystal structure of the cytoplasmic domain of the type I TGFβ receptor in complex with FKBP12. *Cell,* **96**(3), 425-36.

[18] Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., Gelbart, W.M. (1995) Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics,* **139**(3), 1347-58.

[19] Raftery, L.A., Twombly, V., Wharton, K., Gelbart, W.M. (1995) Genetic screens to identify elements of the decapentaplegic signaling pathway in Drosophila. *Genetics,* **139**(1), 241-54.

[20] Savage, C., Das, P., Finelli, A.L., Townsend, S.R., Sun, C.Y., Baird, S.E., Padgett, R.W. (1996) Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. *Proc. Natl. Acad. Sci. USA,* **93**(2), 790-4.

[21] Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H., Kern, S.E. (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science,* **271**(5247), 350-3.

[22] Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.H., Kern, S.E., Massague, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., Smith, J., Thomsen, G.H., Vogelstein, B., Wang, X.F. (1996) Nomenclature: vertebrate mediators of TGFβ family signals. *Cell,* **87**(2), 173.

[23] Riggins, J.G., Kinzler, K., Vogelstein, B and Thiagalingam, S. (1997) Frequency of Smad gene mutations in human cancers. *Cancer Res.,* **57,** 2578-2580.

[24] Riggins, G.J., Thiagalingam, S., Rozenblum, E., Weinstein, C.L., Kern, S.E., Hamilton, S.R., Willson, J.K., Markowitz, S.D., Kinzler, K.W., Vogelstein, B. (1996) Mad-related genes in the human. *Nat. Genet.,* **13**(3), 347-9.

[25] Eppert, K., Scherer, S.W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L.C., Bapat, B., Gallinger, S., Andrulis, I.L., Thomsen, G.H., Wrana, J.L., Attisano, L. (1996) MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell,* **86,** 543-552.

[26] Jonson, T., Gorunova, L., Dawiskiba, S., Andren-Sandberg, A., Stenman, G., ten Dijke, P., Johansson, B., Hoglund, M. (1999) Molecular analyses of the 15q and 18q SMAD genes in pancreatic cancer. *Genes. Chromosomes Cancer,* **24**, 62-71.

[27] Uchida, K., Nagatake, M., Osada, H., Yatabe, Y., Kondo, M., Mitsudomi, T., Matsuda, A., Takahashi, T., Takahashi,

T. (1996) Somatic *in vivo* alterations of the JV18-1 gene at 18q21 in human lung cancers. *Cancer Res.*, **56**, 5583-5585.

[28] Schutte, M., Hruban, R.H., Hedrick, L., Cho, K.R., Nadasdy, G.M., Weinstein, C.L., Bova, G.S., Isaacs, W.B., Cairns, P., Nawroz, H., Sidransky, D., Casero, R.A. Jr., Meltzer, P.S., Hahn, S.A., Kern, S.E. (1996) DPC4 gene in various tumor types. *Cancer Res.*, **56**, 2527-2530.

[29] Yakicier, M.C., Irmak, M.B., Romano, A., Kew, M., Ozturk, M. (1999) Smad2 and Smad4 gene mutations in hepatocellular carcinoma. *Oncogene*, **18**, 4879-83.

[30] Bouras, M., Tabone, E., Bertholon, J., Sommer, P., Bouvier, R., Droz, J.P., Benahmed, M. (2000) A novel SMAD4 gene mutation in seminoma germ cell tumors. *Cancer Res.*, **60**(4), 922-8.

[31] Kim, S. K., Fan, Y., Papadimitrakopoulou, V., Clayman, G., Hittelman, W.N., Hong, W.K., Lotan, R., Mao, L. (1996) DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Res.*, **56**, 2519-2521.

[32] Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.E., Heldin, C.H., ten Dijke, P. (1997) Identification of Smad7, a TGFβ-inducible antagonist of TGFβ signalling. *Nature*, **389**, 631-635.

[33] Watanabe, T.K., Suzuki, M., Omori, Y., Hishigaki, H., Horie, M., Kanemoto, N., Fujiwara, T., Nakamura, Y., Takahashi, T. (1997) Cloning and characterization of a novel member of the human Mad gene family (MADH6). Genomics, **42**, 446-451.

[34] Hata, A., Shi, Y., Massague, J. (1998) TGFβ signaling and cancer: structural and functional consequences of mutations in Smads. *Mol. Med. Today*, **4**(6), 257-62.

[35] Shi, Y., Wang, Y.F., Jayaraman, L., Yang, H., Massague, J., Pavletich, N.P. (1998) Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGFβ signaling. *Cell*, **94**(5), 585-94.

[36] De Caestecker, M.P., Hemmati, P., Larisch-Bloch, S., Ajmera, R., Roberts, A.B., Lechleider, R.J. (1997) Characterization of functional domains within Smad4/DPC4. *J. Biol. Chem.*, **272**(21), 13690-6.

[37] Luo, K., Stroschein, S.L., Wang, W., Chen, D., Martens, E., Zhou, S., Zhou, Q. (1999) The Ski oncoprotein interacts with the Smad proteins to repress TGFβ signaling. *Genes. Dev.*, **13**(17), 2196-206.

[38] Wotton, D., Lo, R.S., Lee, S., Massague, J. (1999) A Smad transcriptional corepressor. *Cell*, **97**, 29–39.

[39] Lo, R.S., Chen, Y.G., Shi, Y., Pavletich, N.P., Massague, J. (1998) The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGFβ receptors. *EMBO. J.*, **17**(4), 996-1005.

[40] Hata, A., Lo, R.S., Wotton, D., Lagna, G., Massague, J. (1997) Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature*, **388**(6637), 82-7.

[41] Zhang, Y., Feng, X., We, R., Derynck, R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGFβ response. *Nature*, **383**(6596), 168-72.

[42] Hoodless, P.A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L., Wrana, J.L. (1996) MADR1, a MAD-related protein that functions in BMP2 signaling pathways. *Cell*, **85**(4), 489-500.

[43] Macias-Silva, M., Abdollah, S., Hoodless, P.A., Pirone, R., Attisano, L., Wrana, J.L. (1996) MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell*, **87**(7), 1215-24.

[44] Souchelnytskyi, S., ten Dijke, P., Miyazono, K., Heldin, C.H. (1996) Phosphorylation of Ser165 in TGFβ type I receptor modulates TGFβ1-induced cellular responses. *EMBO. J.*, **15**(22), 6231-40.

[45] Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., Wrana, J.L. (1997) TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J. Biol. Chem.*, **272**(44), 27678-85.

[46] Masuyama, N., Hanafusa, H., Kusakabe, M., Shibuya, H., Nishida, E. (1999) Identification of two Smad4 proteins in Xenopus. Their common and distinct properties. *J. Biol. Chem.*, **274**, 12163-12170.

[47] Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., Wrana, J.L. (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. *Cell*, **95**(6), 779-91.

[48] Shi, Y., Hata, A., Lo, R.S., Massague, J., Pavletich, N.P. (1997) A structural basis for mutational inactivation of the tumour suppressor Smad4. *Nature*, **388**(6637), 87-93.

[49] Wrana, J., Pawson, T. (1997) Signal transduction. Mad about SMADs. *Nature*, **388**(6637), 28-9.

[50] Qin, B., Lam, S.S., Lin, K. (1999) Crystal structure of a transcriptionally active Smad4 fragment. *Structure*, **7**, 1493-1503.

[51] Kawabata, M., Inoue, H., Hanyu, A., Imamura, T., Miyazono, K. (1998) Smad proteins exist as monomers *in vivo* and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. *EMBO. J.*, **17**(14), 4056-65.

[52] Jayaraman, L., Massague, J. (2001) Distinct oligomeric states of SMAD proteins in the transforming growth factor β pathway. *J Biol. Chem.*, **275**(52), 40710-17.

[53] Wu, J.W., Fairman, R., Penry, J., Shi, Y. (2001) Formation of a stable heterodimer between Smad2 and Smad4. *J. Biol. Chem.*, **276**(23)20688-94.

* Purified Smad2 and Smad4 proteins analyzed by both biochemical and biophysical methods only exhibited a stable heterodimer formation in the presence of an intact MH2 domain of Smad4.

[54] Chacko, B.M., Qin, B., Correia, J.J., Lam, S.S., de Caestecker, M.P., Lin, K. (2001) The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. *Nat. Struct. Biol.*, **8**(3), 248-53.

[55] Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., Gauthier, J.M. (1998) Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.*, **17**, 3091–100.

[56] Jonk, L.J., Itoh, S., Heldin, C.H., ten Dijke, P., Kruijer, W. (1998) Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. *J. Biol. Chem.,* **273**(33), 21145-52.

[57] Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., Kern, S.E. (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell,* **1**, 611–7.

[58] Yagi, K., Goto, D., Hamamoto, T., Takenoshita, S., Kato, M., Miyazono, K. (1999) Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3. *J. Biol. Chem.,* **274**(2), 703-9.

[59] Liu, F., Pouponnot, C., Massague, J. (1997) Dual role of the Smad4/DPC4 tumor suppressor in TGFβ-inducible transcriptional complexes. *Genes. Dev.,* **11**(23), 3157-67.

[60] Das, P., Maduzia, L.L., Wang, H., Finelli, A.L., Cho, S.H., Smith, M.M., Padgett, R.W. (1998) The Drosophila gene Medea demonstrates the requirement for different classes of Smads in dpp signaling. *Development,* **125**(8), 1519-28.

[61] Attisano, L., Wrana, J.L. (2000) Smads as transcriptional co-modulators. *Curr. Opin. Cell. Biol.,* **12**(2), 235-43.

[62] Ten Dijke, P., Miyazono, K., Heldin, C.H. (2000) Signaling inputs converge on nuclear effectors in TGFβ signaling. *Trends Biochem. Sci.,* **25**(2), 64-70.

[63] Chen, X., Rubock, M.J., Whitman, M. (1996) A transcriptional partner for MAD proteins in TGFβ signalling. *Nature,* **383**, 691–6.

[64] Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., Whitman, M. (1997) Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature,* **389**, 85–9.

[65] Labbe, E., Silvestri, C., Hoodless, P.A., Wrana, J.L., Attisano, L. (1998) Smad2 and Smad3 positively and negatively regulate TGFβ-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol. Cell,* **2**(1), 109-20.

[66] Zhang, Y., Feng, X.H., Derynck, R. (1998) Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGFβ-induced transcription. *Nature,* **394**(6696), 909-13.

[67] Zhou, S., Zawel, L., Lengauer, C., Kinzler, K.W., Vogelstein, B. (1998) Characterization of human FAST-1, a TGFβ and activin signal transducer. *Mol. Cell,* **2**(1), 121-7.

[68] Feng, X.H., Zhang, Y., Wu, R.Y., Derynck, R. (1998) The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGFβ-induced transcriptional activation. *Genes. Dev.,* **12**(14), 2153-63.

[69] Janknecht, R., Wells, N.J., Hunter, T. (1998) TGFβ-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes. Dev.,* **12**(14), 2114-9.

[70] Pouponnot, C., Jayaraman, L., Massague, J. (1998) Physical and functional interaction of SMADs and p300/CBP. *J. Biol. Chem.,* **273**(36), 22865-8.

[71] Shen, X., Hu, P.P., Liberati, N.T., Datto, M.B., Frederick, J.P., Wang, X.F. (1998) TGFβ-induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein. *Mol. Biol. Cell,* **9**, 3309-19.

[72] Snowden, A.W., Perkins, N.D. (1998) Cell cycle regulation of the transcriptional coactivators p300 and CREB binding protein. *Biochem. Pharmacol.,* **55**(12), 1947-54.

[73] Xu, L., Glass, C.K., Rosenfeld, M.G. (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.,* **9**, 140-7.

[74] Verschueren, K., Remacle, J.E., Collart, C., Kraft, H., Baker, B.S., Tylzanowski, P., Nelles, L., Wuytens, G., Su, M.T., Bodmer, R., Smith, J.C., Huylebroeck, D. (1999) SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. *J. Biol. Chem.,* **274**, 20489–98.

[75] Wotton, D., Lo, R.S., Swaby, L.A., Massague, J. (1999) Multiple modes of repression by the Smad transcriptional corepressor TGIF. *J. Biol. Chem.,* **274**, 37105–10.

[76] Sun, Y., Liu, X., Eaton, E.N., Lane, W.S., Lodish, H.F., Weinberg, R.A. (1999) Interaction of the Ski oncoprotein with Smad3 regulates TGFβ signaling. *Mol. Cell,* **4**, 499–509.

[77] Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H.F., Weinberg, R.A. (1999) SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. *Proc. Natl. Acad. Sci. USA,* **96**, 12442–7.

[78] Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K., *et al.* (1999) c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. *J. Biol. Chem.,* **274**, 35269–77.

[79] Xu, W., Angelis, K., Danielpour, D., Haddad, M.M., Bischof, O., Campisi, J., Stavnezer, E., Medrano, E.E. (2000) Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type beta transforming growth factor. *Proc. Natl. Acad. Sci. USA,* **97**(11), 5924-9.

[80] Stroschein, S.L., Wang, W., Zhou, S., Zhou, Q., Luo, K. (1999) Negative feedback regulation of TGFβ signaling by the SnoN oncoprotein. *Science,* **286**, 771–4.

[81] Van Grunsven, L.A., Schellens, A., Huylebroeck, D., Verschueren, K. (2001) SIP1 (Smad interacting protein 1) and deltaEF1 (delta-crystallin enhancer binding factor) are structurally similar transcriptional repressors. J. Bone Joint Surg. Am., 83-A Suppl 1 (Pt 1) S40-7.

[82] Hanai, J., Chen, L.F., Kanno, T., Ohtani-Fujita, N., Kim, W.Y., Guo, W.H., Imamura, T., Ishidou, Y., Fukuchi, M., Shi, M.J., Stavnezer, J., Kawabata, M., Miyazono, K., Ito, Y. (1999) Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. *J. Biol. Chem.,* **274**, 31577–82.

[83] Pardali, E., Xie, X.Q., Tsapogas, P., Itoh, S., Arvanitidis, K., Heldin, C.H., ten Dijke, P., Grundstrom, T., Sideras, P. (2000) Smad and AML proteins synergistically confer transforming growth factor beta1 responsiveness to human germ-line IgA genes. *J. Biol. Chem.,* **275**, 3552–60.

[84] Zhang, Y., Derynck, R. (2000) Transcriptional regulation of the transforming growth factor-beta -inducible mouse germ line Ig alpha constant region gene by functional cooperation of Smad, CREB, and AML family members. *J. Biol. Chem.,* **275**(22), 16979-85.

[85] Sano, Y., Harada, J., Tashiro, S., Gotoh-Mandeville, R., Maekawa, T., Ishii, S. (1999) ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. *J. Biol. Chem.*, **274**, 8949–57.

[86] Germain, S., Howell, M., Esslemont, G.M., Hill, C.S. (2000) Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes. Dev.*, **14**, 435–51.

[87] Zhang, Y., Feng, X.H., Derynck, R. (1998) Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGFβ-induced transcription. *Nature*, **394**(6696), 909-13.

[88] Liberati, N.T., Datto, M.B., Frederick, J.P., Shen, X., Wong, C., Rougier-Chapman, E.M., Wang, X.F. (1999) Smads bind directly to the Jun family of AP-1 transcription factors. *Proc. Natl. Acad. Sci. USA*, **96**, 4844–9.

[89] Qing, J., Zhang, Y., Derynck, R. (2000) Structural and functional characterization of the transforming growth factor-beta -induced Smad3/c-Jun transcriptional cooperativity. *J. Biol. Chem.*, **275**(49), 38802-12.

[90] Datta, P.K., Blake, M.C., Moses, H.L. (2000) Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-beta -induced physical and functional interactions between smads and Sp1. *J. Biol. Chem.*, **275**(51), 40014-9.

[91] Feng, X.H., Lin, X., Derynck, R. (2000) Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGFβ. *EMBO. J.*, **19**(19), 5178-93.
*
with a complex of Smad2, Smad3 and Smad4 are involved in the induction of $p15^{INK4B}$.

[92] Pardali, K., Kurisaki, A., Moren, A., ten Dijke, P., Kardassis, D., (2000) Moustakas Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J. Biol. Chem.*, **275**(38), 29244-56.
*
ability of the latter to bind G/C-rich DNA elements and results in upregulation of p21 expression.

[93] Moustakas, A., Kardassis, D. (1998) Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. *Proc. Natl. Acad. Sci. USA*, **95**, 6733–8.

[94] Adnane, J., Bizouarn, F.A., Qian, Y., Hamilton, A.D., Sebti, S.M. (1998) p21(WAF1/CIP1) is upregulated by the geranylgeranyltransferase I inhibitor GGTI-298 through a transforming growth factor beta- and Sp1-responsive element: involvement of the small GTPase rhoA. *Mol. Cell Biol.*, **18**, 6962–70.

[95] Yoshizumi, M., Wang, H., Hsieh, C.M., Sibinga, N.E., Perrella, M.A., Lee, M.E. (1997) Down-regulation of the cyclin A promoter by transforming growth factor-beta1 is associated with a reduction in phosphorylated activating transcription factor-1 and cyclic AMP-responsive element-binding protein. *J. Biol. Chem.*, **272**, 22259–64.

[96] Chen, Y.G., Hata, A., Lo, R.S., Wotton, D., Shi, Y., Pavletich, N., Massague, J. (1998) Determinants of specificity in TGFβ signal transduction. *Genes. Dev.*, **12**, 2144–52.

[97] Vindevoghel, L., Lechleider, R.J., Kon, A., de Caestecker, M.P., Uitto, J., Roberts, A.B., Mauviel, A. (1998) SMAD3/4-dependent transcriptional activation of the human type VII collagen gene (COL7A1) promoter by transforming growth factor beta. *Proc. Natl. Acad. Sci. USA*, **95**, 14769–74.

[98] De Caestecker, M.P., Yahata, T., Wang, D., Parks, W.T., Huang, S., Hill, C.S., Shioda, T., Roberts, A.B., Lechleider, R.J. (2000) The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. *J. Biol. Chem.*, **275**(3), 2115-22.

[99] Shioda, T., Lechleider, R.J., Dunwoodie, S.L., Li, H., Yahata, T., de Caestecker, M.P., Fenner, M.H., Roberts, A.B., Isselbacher, K.J. (1998) Transcriptional activating activity of Smad4: roles of SMAD hetero-oligomerization and enhancement by an associating transactivator. *Proc. Natl. Acad. Sci. USA*, **95**, 9785–90.

[100] Yahata, T., de Caestecker, M.P., Lechleider, R.J., Andriole, S., Roberts, A.B., Isselbacher, K.J., Shioda, T. (2000) The MSG1 non-DNA-binding transactivator binds to the p300/CBP coactivators, enhancing their functional link to the Smad transcription factors. *J. Biol. Chem.*, **275**, 8825–34.

[101] Jakubowiak, A., Pouponnot, C., Berguido, F., Frank, R., Mao, S., Massague, J., Nimer, S.D. (2000) Inhibition of the transforming growth factor beta 1 signaling pathway by the AML1/ETO leukemia-associated fusion protein. *J. Biol. Chem.*, **275**(51), 40282-7.

[102] Nishihara, A., Hanai, J., Imamura, T., Miyazono, K., Kawabata, M. (1999) E1A inhibits transforming growth factor-beta signaling through binding to Smad proteins. *J. Biol. Chem.*, **274**, 28716–23.

[103] Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K., Hirai, H. (1998) The oncoprotein Evi-1 represses TGFβ signalling by inhibiting Smad3. *Nature*, **394**, 92–6.

[104] Kurokawa, M., Mitani, K., Imai, Y., Ogawa, S., Yazaki, Y., Hirai, H. (1998) The t(3;21) fusion product, AML1/Evi-1, interacts with Smad3 and blocks transforming growth factor-beta-mediated growth inhibition of myeloid cells. *Blood*, **92**, 4003–12.

[105] Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., Miyazono, K. (1997) Smad6 inhibits signalling by the TGFβ superfamily. *Nature*, **389**(6651), 622-6.

[106] Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y.Y., Grinnell, B.W., Richardson, M.A., Topper, J.N., Gimbrone, M.A. Jr., Wrana, J.L., Falb, D. (1997) The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Cell*, **89**(7), 1165-73.

[107] Datta, P.K., Chytil, A., Gorska, A.E., Moses, H.L. (1998) Identification of STRAP, a novel WD domain protein in transforming growth factor-beta signaling. *J. Biol. Chem.*, **273**(52), 34671-4.

[108] Bai, S., Shi, X., Yang, X., Cao, X. (2000) Smad6 as a transcriptional corepressor. *J. Biol. Chem.*, **275**(12), 8267-70.

* Smad6 interacts with Hoxc-8 inhibiting Smad1-Hoxc-8 complex formation, thus repressing BMP-signaling in the nucleus.

[109] Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L., Thomsen, G.H. (1999) A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature,* **400**(6745), 687-93.

[110] Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., Wrana, J.L. (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. *Mol. Cell,* **6**, 1365-75.

* Nuclear Smurf2 binds to Smad7 and induces the export of Smad7 to the cytoplasm where it binds to an activated TGFβ receptor and causes degradation of these molecules.

[111] Lo, R.S., Massague, J. (1999) Ubiquitin-dependent degradation of TGFβ-activated smad2. *Nat. Cell Biol.,* **1**, 472-8.

[112] Zimmerman, C.M., Padgett, R.W. (2000) Transforming growth factor beta signaling mediators. *Gene,* **249**, 17-30.

[113] Itoh, S., Itoh, F., Goumans, M.J., Ten Dijke, P. (2000) Signaling of transforming growth factor-beta family members through Smad proteins. *Eur. J. Biochem.,* **267**(24), 6954-67.

[114] Miyazono, K., Ichijo, H., Heldin, C.H. (1993) Transforming growth factor-beta: latent forms, binding proteins and receptors. *Growth Factors,* **8**, 11-22.

[115] Eklov, S., Funa, K., Nordgren, H., Olofsson, A., Kanzaki, T., Miyazono, K., Nilsson, S. (1993) Lack of the latent transforming growth factor beta binding protein in malignant, but not benign prostatic tissue. *Cancer Res.,* **53**(13), 3193-7.

[116] Saharinen, J., Taipale, J., Monni, O., Keski-Oja, J. (1998) Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4. *J. Biol. Chem.,* **273**(29), 18459-69.

[117] Ribeiro, S.M., Poczatek, M., Schultz-Cherry, S., Villain, M, , Murphy-Ullrich J.E. (1999) The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta. *J. Biol. Chem.,* **274**(19), 13586-93.

[118] Miller, D.W., Graulich, W., Karges, B., Stahl, S., Ernst, M., Ramaswamy, A., Sedlacek, H.H., Muller, R., Adamkiewicz, J. (1999) Elevated expression of endoglin, a component of the TGFβ-receptor complex, correlates with proliferation of tumor endothelial cells. *Int. J. Cancer,* **81**(4), 568-72.

[119] Kumar, S., Ghellal, A., Li, C., Byrne, G., Haboubi, N., Wang, J.M., Bundred, N. (1999) Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res.,* **59**(4), 856-61.

[120] Li, C., Guo, B., Bernabeu, C., Kumar, S. (2001) Angiogenesis in breast cancer: The role of transforming growth factor beta and CD105. *Microsc. Res. Tech.,* **52**(4), 437-49.

[121] Roberts, A.B., Piek, E and de Caestecker, M.P. (2001) Response: re: role of transforming growth factor-beta signaling in cancer. *J. Natl. Can. Inst.,* **93**, 555-7.

[122] Kaname, S., Ruoslahti, E. (1996) Betaglycan has multiple binding sites for transforming growth factor-beta 1. *Biochem. J.,* **315** (Pt 3), 815-20.

[123] Lopez-Casillas, F., Wrana, J.L., Massague, J. (1993) Betaglycan presents ligand to the TGFβ signaling receptor. *Cell,* **73**(7)1435-44.

[124] Sankar, S., Mahooti-Brooks, N., Centrella, M., McCarthy, T.L., Madri, J.A. (1995) Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor beta 2. *J. Biol. Chem.,* **270**(22), 13567-72.

[125] Sporn, M.B., Roberts, A.B. (1985) Autocrine growth factors and cancer. *Nature,* **313**, 745-747.

[126] Roberts, A.B., Sporn, M.B. (1993) Physiological actions and clinical applications of transforming growth factor-beta (TGFβ). *Growth Factors,* **8**, 1-9.

[127] Kim, I. Y., Ahn, H.J., Zelner, D.J., Shaw, J., Sensibar, J., Kim, J., Kato, M., Lee, C. (1996) Genetic change in transforming growth factor β (TGFβ) receptor type I gene correlates with insensitivity to TGFβ1 in human prostate cancer cells. *Cancer Res.,* **56**, 44-48.

[128] Kimchi, A., Wang, X., Weinberg, R., Cheifetzn, S., Massague, J. (1988) Absence of TGFβ receptors and growth inhibitory responses in retinoblastoma cells. *Science,* **240**, 196-199.

[129] Park, K., Kim, S., Bang, Y.J., Park, J.G., Kim, N.K., Roberts, A.B., Sporn, M.B. (1994) Genetic changes in the transforming growth factor beta (TGFβ) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGFβ. *Proc. Natl. Acad. Sci. USA,* **91**, 8772-8776.

[130] Sun, L., Wu, G., Willson, J., Zborowska, E., Yang, J., Rajkarunanayake, I., Wang, J., Centry, L.E., Wang, X.F., Brattain, G. (1994) Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J. Biol. Chem.,* **269**, 26449-26455.

[131] Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L.Z., Lutterbaough, J., Fan, R.S., Zborowska, E., Kinzler, K.W., Vogelstein, B., Brattain, M.G., Willson, J.K.V. (1995) Inactivation of the type II TGFβ receptor in colon cancer cells with microsatellite instability. *Science,* **268**, 1336-1338.

[132] Parsons, R., Myeroff, L.L., Liu, B., Willson, J.K., Markowitz, S.D., Kinzler, K.W., Vogelstein, B. (1995) Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res.,* **55**, 5548-5550.

[133] Grady, W.M., Myeroff, L.L., Swinler, S.E., Rajaput, A., Thiagalingam, S., Lutterbaugh, J.D., Neumann, A., Brattain, M.G., Chang, J., Kim, S.J., Kinzler, K.W., Vogelstein, B., Willson, J.K.V., Markowitz, S. (1999) Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res.,* **59**, 320-324.

[134] Izumoto, S., Arita, N., Ohnishi, T., Hiraga, S., Taki, T., Tomita, N., Ohue, M., Hayakawa, T. (1997) Microsatellite instability and mutated type II transforming growth factor-beta receptor gene in gliomas. *Cancer Lett.,* **112**(2), 251-6.

[135] Lu, S.L., Kawabata, M., Imamura, T., Miyazono, K., Yuasa, Y. (1999) Two divergent signaling pathways for TGFβ separated by a mutation of its type II receptor gene. *Biochem. Biophys. Res. Commun.*, **259**, 385-90.

[136] Wang, D., Kanuma, T., Mizunuma, H., Takama, F., Ibuki, Y., Wake, N., Mogi, A., Shitara, Y., Takenoshita, S. (2000) Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. *Cancer Res.*, **60**(16), 4507-12.

[137] Chen, T., Carter, D., Garrigue-Antar, L., Reiss, M. (1998) Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res.*, Nov 1, **58**(21), 4805-10.

[138] Goggins, M., Shekher, M., Turnacioglu, K., Yeo, C.J, Hruban, R.H., Kern, S.E. (1998) Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Res.*, **58**(23), 5329-32.

[139] Schiemann, W.P., Pfeifer, W.M., Levi, E., Kadin, M.E., Lodish, H.F. (1999) A deletion in the gene for transforming growth factor beta type I receptor abolishes growth regulation by transforming growth factor beta in a cutaneous T-cell lymphoma. *Blood*, **94**(8), 2854-61.

[140] Kang, S.H., Bang, Y.J., Im, Y.H., Yang, H.K., Lee, D.A., Lee, H.Y., Lee, H.S., Kim, N.K., Kim, S.J. (1999) Transcriptional repression of the transforming growth factor-beta type I receptor gene by DNA methylation results in the development of TGFβ resistance in human gastric cancer. *Oncogene*, Dec 2, **18**(51), 7280-6.

[141] Nagatake, M., Takagi, Y., Osada, H., Uchida, K., Mitsudomi, T., Saji, S., Shimokawa, K., Takahashi, T. (1996) Aberrant hypermethylation at the bcl-2 locus at 18q21 in human lung cancers. *Cancer Res.*, **56**, 2718-2720.

[142] Thiagalingam, S., Lengauer, C., Leach, F.S., Schutte, M., Hahn, S.A., Overhauser, J., Willson, J.K.V., Markowitz, S., Hamilton, S.R., Kern, S.E., Kinzler, K.W., Vogelstein, B. (1996) Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nature Genet.*, **13**, 343-346.

[143] Kern, S.E., Fearon, E.R., Tersmette, K.W., Enterline, J.P., Leppert, M., Nakamura, Y., White, R., Vogelstein, B., Hamilton, S.R. (1989) Clinical and pathological associations with allelic loss in colorectal carcinoma. *JAMA*, **261**, 3099-103.

[144] Jen, J., Kim, H., Piantadosi, S., Liu, Z.F., Levitt, R.C., Sistonen, P., Kinzler, K.W., Vogelstein, B., Hamilton, S.R. (1994) Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N. Engl. J. Med.*, Jul 28, **331**(4), 213-21.

[145] Yanagisawa, K., Uchida, K., Nagatake, M., Masuda, A., Sugiyama, M., Saito, T., Yamaki, K., Takahashi, T., Osada, H. (2000) Heterogeneities in the biological and biochemical functions of Smad2 and Smad4 mutants naturally occurring in human lung cancers. *Oncogene*, **19**, 2305-11.

[146] Funaba, M., Mathews, L.S. (2000) Identification and characterization of constitutively active Smad2 mutants: evaluation of formation of Smad complex and subcellular distribution. *Mol. Endocrinol.*, **14**(10), 1583-91.

[147] Prunier, C., Ferrand, N., Frottier, B., Pessah, M., Atfi, A. (2001) Mechanism for mutational inactivation of the tumor suppressor smad2. *Mol. Cell. Biol.*, **21**, 3302-13.

[148] Moren, A., Itoh, S., Moustakas, A., Dijke, P., Heldin, C.H. (2000) Functional consequences of tumorigenic missense mutations in the amino-terminal domain of Smad4. *Oncogene*, **19**(38), 4396-404.

[149] Xu, J., Attisano, L. (2000) Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor beta signaling by targeting Smads to the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA*, **97**, 4820-5.

[150] Koyama, M., Ito, M., Nagai, H., Emi, M., Moriyama, Y. (1999) Inactivation of both alleles of the DPC4/SMAD4 gene in advanced colorectal cancers: identification of seven novel somatic mutations in tumors from Japanese patients. *Mutat. Res.*, **406**, 71-7.

[151] Takakura, S., Okamoto, A., Saito, M., Yasuhara, T., Shinozaki, H., Isonishi, S., Yoshimura, T., Ohtake, Y., Ochiai, K., Tanaka, T. (1999) Allelic imbalance in chromosome band 18q21 and SMAD4 mutations in ovarian cancers. *Genes Chromosomes Cancer*, **24**(3), 264-71.

[152] MacGrogan, D., Pegram, M., Slamon, D., Bookstein, R. (1997) Comparative mutational analysis of DPC4 (Smad4) in prostatic and colorectal carcinomas. *Oncogene*, **15**(9), 1111-4.

[153] Imai, Y., Kurokawa, M., Izutsu, K., Hangaishi, A., Maki, K., Ogawa, S., Chiba, S., Mitani, K., Hirai, H. (2001) Mutations of the Smad4 gene in acute myelogeneous leukemia and their functional implications in leukemogenesis. *Oncogene*, **20**(1), 88-96.

[154] Miyaki, M., Iijima, T., Konishi, M., Sakai, K., Ishii, A., Yasuno, M., Hishima, T., Koike, M., Shitara, N., Iwama, T., Utsunomiya, J., Kuroki, T., Mori, T. (1999) Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene*, **18**, 3098-3103.

[155] Bartsch, D., Hahn, S.A., Danichevski, K.D., Ramaswamy, A., Bastian, D., Galehdari, H., Barth, P., Schmiegel, W., Simon, B., Rothmund, M. (1999) Mutations of the DPC4/Smad4 gene in neuroendocrine pancreatic tumors. *Oncogene*, **18**, 2367-2371.

[156] Hoque, A.T., Hahn, S.A., Schutte, M., Kern, S.E. (1997) DPC4 gene mutation in colitis associated neoplasia. *Gut.*, **40**, 120-122.

[157] Kim, S. K., Fan, Y., Papadimitrakopoulou, V., Clayman, G., Hittelman, W.N., Hong, W.K., Lotan, R., Mao, L. (1996) DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Res.*, **56**, 2519-2521.

[158] Takagi, Y., Koumura, H., Futamura, M., Aoki, S., Ymaguchi, K., Kida, H., Tanemura, H., Shimokawa, K., Saji, S. (1998) Somatic alterations of the SMAD-2 gene in human colorectal cancers. *Br. J. Cancer*, **78**, 1152-5.

[159] Kleeff, J., Ishiwata, T., Maruyama, H., Friess, H., Truong, P., Buchler, M.W., Falb, D., Korc, M. (1999) The TGFβ signaling inhibitor Smad7 enhances tumorigenicity in pancreatic cancer. *Oncogene*, **18**(39), 5363-72.

[160] Wick, W., Petersen, I., Schmutzler, R.K., Wolfarth, B., Lenartz, D., Bierhoff, E., Hummerich, J., Muller, D.J., Stangl, A.P., Schramm, J., Wiestler, O.D., von, Deimling. A. (1996) Evidence for a novel tumor suppressor gene on chromosome 15 associated with progression to a metastatic stage in breast cancer. *Oncogene*, **12**(5), 973-8.

[161] Gorunova, L., Hoglund, M., Andren-Sandberg, A., Dawiskiba, S., Jin, Y., Mitelman, F., Johansson, B. (1998) Cytogenetic analysis of pancreatic carcinomas: intratumor heterogeneity and nonrandom pattern of chromosome aberrations. *Genes Chromosomes Cancer*, **23**, 81-99.

[162] Hahn, S.A., Kern, S.E. (1995) Molecular genetics of exocrine pancreatic neoplasms. *Surg. Clin. North Am.*, **75**, 857-69.

[163] Arai, T., Akiyama, Y., Okabe, S., Ando, M., Endo, M., Yuasa, Y. (1998) Genomic structure of the human Smad3 gene and its infrequent alterations in colorectal cancers. *Cancer Lett.*, **122**, 157-63.

[164] Bevan, S., Woodford-Richens, K., Rozen, P., Eng, C., Young, J., Dunlop, M., Neale, K., Phillips, R., Markie, D., Rodriguez-Bigas, M., Leggett, B., Sheridan, E., Hodgson, S., Iwama, T., Eccles, D., Bodmer, W., Houlston, R., Tomlinson, I. (1999) Screening SMAD1, SMAD2, SMAD3, and SMAD5 for germline mutations in juvenile polyposis syndrome. *Gut.*, **45**, 406-8.

[165] Roth, S., Sistonen, P., Salovaara, R., Hemminki, A., Loukola, A., Johansson, M., Avizienyte, E., Cleary, K.A., Lynch, P., Amos, C.I., Kristo, P., Mecklin, J.P., Kellokumpu, I., Jarvinen, H., Aaltonen, L.A. (1999) SMAD genes in juvenile polyposis. *Genes Chromosomes Cancer*, **26**, 54-61.

[166] Wang, D., Kanuma, T., Takama, F., Mizumuma, H., Ibuki, Y., Wake, N., Mogi, A., Shitara, Y., Hagiwara, K., Takenoshita, S. (1999) Mutation analysis of the Smad3 gene in human ovarian cancers. *Int. J. Oncol.*, **15**, 949-53.

[167] Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A., Leppert, M., Nakamura, Y., White, R., Smith, A.M.M., Boss, J.L. (1988) Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525-532.

[168] Yogota, J., Sugimura, T. (1993) Multiple steps in carcinogenesis involving alterations of multiple tumor suppressor genes. *FASEB. J.*, **7**, 920-925.

[169] Ueda, T., Komiya, A., Emi, M., Suzuki, H., Shiraishi, T., Yatani, R., Masai, M., Yasuda, K., Ito, H. (1997) Allelic losses on 18q21 are associated with progression and metastasis in human prostate cancer. *Genes Chromosom. Cancer*, **20**, 140-147.

[170] Amendt, C., Schirmacher, P., Weber, H., Blessing, M. (1998) Expression of a dominant negative type II TGFβ receptor in mouse skin results in an increase in carcinoma incidence and an acceleration of carcinoma development. *Oncogene*, **17**, 25-34.

[171] Takei, K., Kohno, T., Hamada, K., Takita, J., Noguchi, M., Matsuno, Y., Hirohashi, S., Uezato, H., Yokota, J. (1998) A novel tumor suppressor locus on chromosome 18q involved in the development of human lung cancer. *Cancer Res.*, **58**, 3700-3705.

[172] Sherr, C.J., Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.*, **13**, 1501–1512.

[173] Hannon, G.J., Beach, D. (1994) p15INK4B is a potential effector of TGFβ-induced cell cycle arrest. *Nature*, **371**, 257–261.

[174] Sandhu, C., Garbe, J., Bhattacharya, N., Daksis, J., Pan, C.H., Yaswen, P., Koh, J., Slingerland, J.M., Stampfer, M.R. (1997) Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. *Mol. Cell. Biol.*, **17**, 2458–2467.

[175] Li, J.M., Nichols, M.A., Chandrasekharan, S., Xiong, Y., Wang, X.F. (1995) Transforming growth factor beta activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site. *J. Biol. Chem.*, **270**(45), 26750-3.

[176] Rich, J.N., Zhang, M., Datto, M.B., Bigner, D.D., Wang, X.F. (1999) Transforming growth factor-beta-mediated p15(INK4B) induction and growth inhibition in astrocytes is SMAD3-dependent and a pathway prominently altered in human glioma cell lines. *J. Biol. Chem.*, **274**(49), 35053-8.

[177] Reynisdóttir, I., Polyak, K., Iavarone, A., Massagué, J. (1995) Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arest in response to TGFβ *Genes Dev.*, **9**, 1831–1845.

[178] Reynisdóttir, I., Massagué, J. (1997) The subcellular location of $p15^{Ink4b}$ and $p27^{Kip1}$ coordinate their inhibitory interactions with cdk4 and cdk2. *Genes Dev.*, **11**, 492–503.

[179] Rorke, E.A., Zhang, D., Choo, C.K., Eckert, R.L., Jacobberger, J.W. (2000) TGFβ-mediated cell cycle arrest of HPV16-immortalized human ectocervical cells correlates with decreased E6/E7 mRNA and increased p53 and p21(WAF-1) expression. *Exp. Cell Res.*, **259**(1), 149-57.

[180] Yoo, Y.D., Choi, J.Y., Lee, S.J., Kim, J.S., Min, B.R., Lee, YI, Kang, Y.K. (1999) TGFβ-induced cell-cycle arrest through the p21(WAF1/CIP1)-G1 cyclin/Cdks-p130 pathway in gastric-carcinoma cells. *Int. J. Cancer*, **83**(4), 512-7.

[181] Datto, M.B., Hu, P.P., Kowalik, T.F., Yingling, J., Wang, X.F. (1997) The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B. *Mol. Cell Biol.*, **17**(4), 2030-7.

[182] Nakayama, K., Ishida, M., Shirame, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y. and Nakayama, K. (1996) Mice lacking p27Kip1 display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell*, **85**, 707–720.

[183] Latres, E., Malumbres, M., Sotillo, R., Martin, J., Ortega, S., Martin-Caballero, J., Flores, J.M., Cordon-Cardo, C., (2000) Barbacid, M. Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *EMBO. J.*, **19**, 3496–3506.

[184] Iavarone, A., Massagué, J. (1997) Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGFβ in cells lacking the CDK inhibitor p15. *Nature*, **387**, 417–422.

[185] Kang, S.H., Bang, Y.J., Jong, H.S., Seo, J.Y., Kim, N.K., Kim, S.J. (1999) Rapid induction of p21WAF1 but delayed

down-regulation of Cdc25A in the TGFβ-induced cell cycle arrest of gastric carcinoma cells. *Br. J. Cancer*, **80**(8), 1144-9.

[186] Dang, C.V. (1999) c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.*, **19**, 1–11.

[187] Alexandrow, M.G., Moses, H.L. (1995) Transforming growth factor β and cell cycle regulation. *Cancer Res.*, **55**, 1452–1457.

[188] Warner, B.J., Blain, S.W., Seoane, J., Massague, J. (1999) Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G1 arrest pathway. *Mol. Cell Biol.*, **19**, 5913–5922.

[189] Claassen, G.F., Hann, S.R. (2000) A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta-induced cell-cycle arrest. *Proc. Natl. Acad. Sci. USA*, **97**, 9498–9503.

[190] Galaktionov, K., Chen, X., (1996) Beach, D. Cdc25 cell cycle phosphatase as a target of c-myc. *Nature*, **382**, 511–517.

[191] Chen, C.R., Kang, Y., Massague, J. (2001) Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc. Natl. Acad. Sci. USA*, **98**(3), 992-9.

[192] Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., Massague, J. (2001) TGFβ influences Myc, Miz-1 and Smad to control the CDK inhibitor $p15^{INK4b}$. *Nature Cell Biol.*, **3**, 400-408.

[193] Graham, A., Koentges, G., Lumsden, A. (1996) Neural crest apoptosis and the establishment of craniofacial pattern: an honorable death. *Mol. Cell Neurosci.*, **8**, 76–83.

[194] Zou, H., Choe, K.M., Lu, Y., Massague, J., Niswander, L. (1997) BMP signaling and vertebrate limb development. *Cold Spring Harb. Symp. Quant. Biol.*, **62**, 269–272.

[195] Nguyen, A.V., Pollard, J.W. (2000) Transforming growth factor β3 induces cell death during the first stage of mammary gland involution. *Development*, **127**, 3107–3118.

[196] Landstrom, M., Heldin, N.E., Bu, S., Hermansson, A., Itoh, S., ten Dijke, P., Heldin, C.H. (2000) Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells. *Curr. Biol.*, **10**(9), 535-8.

[197] Lallemand, F., Mazars, A., Prunier, C., Bertrand, F., Kornprost, M., Gallea, S., Roman-Roman, S., Cherqui, G., Atfi, A. (2001) Smad7 inhibits the survival nuclear factor kappaB and potentiates apoptosis in epithelial cells. *Oncogene*, **20**(7), 879-84.

* The inhibition of the survival factor NF-κB by Smad7 potentiates various forms of cell death.

[198] Yamamura, Y., Hua, X., Bergelson, S., Lodish, H.F. (2000) Critical role of Smads and AP-1 complex in transforming growth factor-beta -dependent apoptosis. *J. Biol. Chem.*, **275**(46), 36295-302.

* Smad3 and Smad4 form a multiprotein complex with JunD/FosB during induction of apoptosis by TGFβ1.

[199] Chen, R.H., Chang, T.Y. (1997) Involvement of caspase family proteases in transforming growth factor-beta induced apoptosis. *Cell Growth Differ.*, **8**, 821–827.

[200] Chipuk, J.E., Bhat, M., Hsing, A.Y., Ma, J., Danielpour, D. (2001) Bcl-xL blocks TGFβ 1-induced apoptosis by inhibiting cytochrome C release and not by directly antagonizing Apaf-1-dependent caspase activation in prostate epithelial cells. *J. Biol. Chem.*, **276**(28), 26614-21.

[201] Herrera, B., Alvarez, A.M., Sanchez, A., Fernandez, M., Roncero, C., Benito, M., Fabregat, I. (2001) Reactive oxygen species (ROS) mediates the mitochondrial-dependent apoptosis induced by transforming growth factor (beta) in fetal hepatocytes. *FASEB. J.*, **15**(3), 741-51.

[202] Akhurst, R.J., Balmain, A. (1999) Genetic events and the role of TGFβ in epithelial tumour progression. *J. Pathol.*, **187**, 82–90.

[203] Denhardt, D.T. (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signaling. *Biochem. J.*, **318**(Pt 3), 729–47.

[204] Dumont, N. (1999) Genetic and epigenetic contributions to colorectal cancer. *APMIS*, **107**, 711–22.

[205] Hannon, G.J., Beach, D. (1994) p15INK4B is a potential effector of TGFβ-induced cell cycle arrest. *Nature*, **371**, 257–261.

[206] Calonge, M.J., Massague, J. (1999) Smad4/DPC4 silencing and hyperactive Ras jointly disrupt transforming growth factor-beta antiproliferative responses in colon cancer cells. *J. Biol. Chem.*, **274**, 33637–43.

[207] Lo, R.S., Wotton, D., Massague, J. (2001) Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *EMBO. J.*, **20**(1-2), 128-36.

* Phosphorylation of TGIF at two Erk-Map kinase sites increases TGIF stabilization, which favors Smad2-TGIF co-repressor complex formation.

[208] Oft, M., Heider, K.H., Beug, H. (1998) TGFβ signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr. Biol.*, **8**, 1243-1252.

[209] Yin, J.J., Selander, K., Chirgwin, J.M., Dallas, M., Grubbs, B.G., Rotraud, W., Massague, J., Mundy, G.R., Guise, T.A. (1999) TGFβ signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J. Clin. Invest*, **103**, 197-206.

[210] De Caestecker, M.P., Parks, W.T., Frank, C.J., Castagnino, P., Bottaro, D.P., Roberts, A.B., Lechleider, R.J. (1998) Smad2 transduces common signals from receptor serine–threonine and tyrosine kinases. *Genes Dev.*, **12**, 1587–92.

[211] Kretzschmar, M., Doody, J., Timokhina, I., Massague, J. (1999) A mechanism of repression of TGFβ/ Smad signaling by oncogenic Ras. *Genes Dev.*, **13**, 804–16.

[212] Atfi, A., Djelloul, S., Chastre, E., Davis, R., Gespach, C. (1997) Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. *J. Biol. Chem.*, **272**, 1429–32.

[213] Hocevar, B.A., Brown, T.L., Howe, P.H. (1999) TGFβ induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO. J.*, **18**, 1345–56.

[214] Osaki, M., Tsukazaki, T., Yonekura, A., Miyazaki, Y., Iwasaki, K., Shindo, H., Yamashita, S. (1999) Regulation of c-fos gene induction and mitogenic effect of transforming growth factor-beta1 in rat articular chondrocyte. *Endocr J.*, **46**(2), 253-61.

[215] Mulder, K.M. (2000) Role of Ras and Mapks in TGFβ signaling. *Cytokine Growth Factor Rev.*, **11**, 23–35.

[216] Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K., Nishida, E. (1999) Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J. Biol. Chem.*, **274**, 27161–7.

[217] Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E., Matsumoto, K. (1996) TAB1: an activator of the TAK1 MAPKKK in TGFβ signal transduction. *Science*, **272**, 1179–82.

[218] Zhou, G., Lee, S.C., Yao, Z., Tan, T.H. (1999) Hematopoietic progenitor kinase 1 is a component of transforming growth factor beta-induced c-Jun N-terminal kinase signaling cascade. *J. Biol. Chem.*, **274**, 13133–8.

[219] Wong, C., Rougier-Chapman, E.M., Frederick, J.P., Datto, M.B., Liberati, N.T., Li, J.M., Wang, X.F. (1999) Smad3–Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. *Mol. Cell Biol.*, **19**, 1821–30.

[220] Brown, J.D., DiChiara, M.R., Anderson, K.R., Gimbrone, M.A. Jr., Topper, J.N. (1999) MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. *J. Biol. Chem.*, **274**, 8797–805.

[221] Ulloa, L., Doody, J., Massague, J. (1999) Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature*, **397**, 710–3.

[222] Bitzer, M., von Gersdorff, G., Liang, D., Dominguez-Rosales, A., Beg, A.A., Rojkind, M., Bottinger, E.P. (2000) A mechanism of suppression of TGFβ/SMAD signaling by NF-kappa B/RelA. *Genes Dev.* **14**, 187–97.

* The activation of Smad7 by NF-κB results in suppression of TGFβ/SMAD signaling.

[223] Cui, W., Fowlis, D.J., Bryson, S., Duffie, E., Ireland, H., Balmain, A., Akhurst, R.J. (1996) TGFβ1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell*, **86**, 531-542.

[224] Fynan, T.M., Reiss, M. (1993) Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit. Rev. Oncog.*, **4**, 493-540.

[225] Cotran, R.S., Kumar, V., Collins, T. (1999) Pathologic basis of disease. W.B. Saunders, Philadelphia, Pennsylvania.

[226] Steiner, M.S., Zhou, Z.Z., Tonb, D.C., Barrack, E.R. (1994) Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology*, **135**, 2240-2247.

[227] Dalal, B.I., Keown, P.A., Greenbery, A.H. (1993) Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *Am. J. Pathol.*, **143**, 381-389.

[228] Walker, R.A., Dearing, S.J., Gallacher, B. (1994) Relationship of transforming growth factor beta 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma. *Br. J. Cancer*, **69**, 1160-1165.

[229] Sargent, E.R., Gomella, L.G., Wade, T.P., Ewing, M.W., Kasid, A., Linehan, W.M. (1989) Expression of mRNA for transforming growth factors-alpha and -beta and secretion of transforming growth factor-beta by renal cell carcinoma cell lines. *Cancer Commun.*, **1**, 317-322.

[230] Sieweke, M.H., Bissell, M.J. (1994) The tumor-promoting effect of wounding: a possible role for TGFβ-induced stromal alterations. *Crit. Rev. Oncog.*, **5**, 297-311.

[231] Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., Reichmann, E. (1996) TGFβ1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev.*, **10**, 2462-2477.

[232] Massague, J., Chen, Y.G. (2000) Controlling TGFβ signaling. *Genes Dev.*, **14**, 627-644.

** Regulation of TGFβ signaling at the level of various players of the pathway as well as the integration of the events with other signaling networks is discussed.

[233] Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.*, **1**, 27-31.

[234] Pepper, M.S., Vassalli, J.D., Orci, L., Montesano, R. (1993) Biphasic effect of transforming growth factor-beta 1 on *in vitro* angiogenesis. *Exp. Cell Res.*, **204**, 356-363.

[235] Gajdusek, C.M., Luo, Z., Mayberg, M.R. (1993) Basic fibroblast growth factor and transforming growth factor beta-1: synergistic mediators of angiogenesis *in vitro*. *J. Cell Physiol.*, **157**, 133-144.

[236] Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., Fauci, A.S. (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Pro. Natl. Acad. Sci.*, **83**, 4167-4171.

[237] Dickson, M.C., Martin, J.S., Cousins, F.M., Kulkarni, A.B., Karlsson, S., Akhurst, R.J. (1995) Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development*, **121**(6), 1845-54

[238] Pepper, M.S. (1997) Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokines Growth Factor Rev.*, **8**, 21-43.

[239] Oh, S.P., Seki, T., Goss, K.A., Imamura, T., Yi, Y., Donahoe, P.K., Li, L., Miyazono, K., Dijke, Pt., Kim, S., Li, E. (2000) Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Pro. Natl. Acad. Sci.*, **97**, 2626-2631.

** TGFβ regulates ALK1 and ALK5 signaling pathways and functions in determining vascular endothelial properties during angiogenesis.

[240] Derbyshire, E., Thrope, P. (1997) Tumour Angiogenesis. Oxford University Pressi, Oxford, England.

[241] Wang, J.M., Kumar, S., Pye, D., van Agthoven, A., Krupinski, J., Hunter, R.D. (1993) A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. *Int. J. Cancer,* **54**, 363-370.

[242] Wang, J.M., Kumar, S., Pye, D., Haboubi, N., Nakib, L. (1994) Breast carcinoma: comparative study of tumor vasculature using two endothelial cell markers. *J. Natl. Cancer Inst.*, **6**, 386-388.

[243] Li, D.Y., Sorensen, L.K., Brook, B.S., Urness, L.D., Davis, E.C., Taylor, D.G. (1999) Defective angiogenesis in mice lacking endoglin. *Science*, **284**, 1534-1537.

[244] Schwarte-Waldhoff, I., Volpert, O.V., Bouck, N.P., Sipos, B., Hahn, S.A., Klein-Scory, S., Luttges, J., Kloppel, G., Graeven, U., Eilert-Micus, C., Hintelmann, A., Schmiegel, W. (2000) Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Pro. Natl. Acad. Sci.*, **97**, 9624-9629.

** Smad4 mediates angiogenic suppression *via* down-regulation of VEGF and upregulation of TSP-1.

[245] Roberts, A.B., Sporn, M.B. (1990) Handbook of Experimental Pharmacology: Peptide Growth Factors and Their Receptors I. Springer-Verlag, Berlin, Germany.

[246] Sodex, J., Overall, C.M. (1992) Matrix metalloproteinases in periodontal tissue remodelling. *Matrix*, **1**, 352-362.

[247] Matrisian, L.M., Ganser, G.L., Kerr, L.D., Pelton, R.W., Wood, L.D. (1992) Negative regulation of gene expression by TGFβ. *Mol. Reprod. Dev.*, **32**, 111-120.

[248] Sehgal, I., Baley, P.A., Thompson, T.C. (1996) Transforming growth factor beta1 stimulates contrasting responses in metastatic versus primary mouse prostate cancer-derived cell lines *in vitro*. *Cancer Res.*, **56**, 3359-65.

[249] Bajou, K., Noel, A., Gerard, R.D. (1998) Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nature Med.*, **4**, 923-928.

[250] Bernhard, E.J., Gruber, S.B., Muschel, R.J. (1994) Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc. Natl. Acad. Sci.*, **91**, 4293-4297.

[251] Welch, D.R., Fabra, A., Nakajima, M. (1990) Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc. Natl. Acad. Sci.*, **87**, 7678-7682.

[252] Samuel, S.K., Hurta, R.A.R., Kondaiah, P., Khalil, N., Turley, E.A., Wright, J.A., Greenbergy, A.H. (1992) Autocrine induction of tumor protease production and invasion by a metallothionein-regulated TGFβ 1 (Ser223, 225). *EMBO. J.*, **11**, 1599-1605.

[253] Stearns, M.E., Stearns, M. (1993) Autocrine factors, type IV collagenase secretion and prostatic cancer cell invasion. *Cancer Metastasis Rev.*, **12**, 39-52.

[254] Chen, R., Ebner, R., Derynck, R. (1993) Inactivation of the type II receptor reveals two receptor pathways for the diverse TGFβ activities. *Science*, **260**, 1335-1338.

[255] Lehmann, K., Janda, E., Pierreux, C.E., Rytomaa, M., Schulze, A., McMahon, M., Hill, C.S., Beug, H., Downward, J. (2000) Raf induces TGFβ production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. *Genes Dev.*, **14**(20), 2610-22.

[256] Piek, E., Moustakas, A., Kurisaki, A., Heldin, C.H., ten Dijke, P. (1999) TGFβ type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J. Cell Sci.*, **112**, 4557-68.

[257] Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., Detmar, M. (2001) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.*, 7(2), 192-8.

[258] Stacker, S.A., Caesar, C., Baldwin, M.E., Thornton, G.E., Williams, R.A., Prevo, R., Jackson, D.G., Nishikawa, S., Kubo, H., Achen, M.G. (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat. Med.*, 7(2), 186-91.

[259] Plate, K. (2001) From angiogenesis to lymphangiogenesis. *Nature Med.*, 7(2), 151-152.

** Vascular endothelial growth factors (VEGFs) are involved in both angiogenesis and lymphangiogenesis, which contribute to hematogenous and lymphatic metastasis, respectively.

[260] Kerhrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M.B., Fauci, A.S. (1986) Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.*, **163**, 1037-50.

[261] Fontana, A., Constam, D.B., Frei, K., Malipiero, U., Pfister, H.W. (1992) Modulation of the immune response by transforming growth factor beta. *Int. Arch. Allergy Immunol.*, 99(1), 1-7.

[262] Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., Taga, T. (1999) Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science*, **284**, 479-82.

[263] Cerwenka, A., Swain, S.L. (1999) TGFβ1: immunosuppressant and viability factor for T lymphocytes. *Microbes & Infection*, **1**(15), 1291-1296.

[264] Wahl, S.M., Orenstein, J.M., Chen, W. (2000) TGFβ influences the life and death decisions of T lymphocytes.*Cytokine Growth Factor Rev.*, **11**, 71-79.

[265] Letterio, J.J., Roberts, A.B. (1996) Transforming growth factor-beta1-deficient mice: identification of isoform-specific activities *in vivo*. *J. Leukoc Biol.*, **59**(6), 769-74.

[266] Letterio, J.J. (2000) Murine models define the role of TGFβ as a master regulator of immune cell function. *Cytokine Growth Factor Rev.*, **11**(1-2), 81-7.

[267] Weinstein, M., Yang, X., Deng, C. (2000) Functions of mammalian Smad genes as revealed by targeted gene disruption in mice. *Cytokine & Growth Factor Reviews*, **11**(1-2), 49-58.

[268] Abbas, A.K., Lichtman, A.H., Pober, J.S. (1994) *Cellular and Molecular Immunology.* (2nd edition) Chapter **18**, 357-375.

[269] Echchakir, H., Bagot, M., Dorothee, G., Martinvalet, D., Le Gouvello, S., Boumsell, L., Chouaib, S., Bensussan, A., Mami-Chouaib, F. (2000) Cutaneous T cell lymphoma reactive CD4+ cytotoxic T lymphocyte clones display a Th1 cytokine profile and use a fas-independent pathway for specific tumor cell lysis. *J. Invest. Dermatol.*, **115**(1), 74-80.

[270] Asselin-Paturel, C., Pardoux, C., Gay, F., Chouaib, S. (1998) Failure of TGFβ1 and IL-12 to regulate human FasL and mTNF alloreactive cytotoxic T-cell pathways. *Tissue Antigens*, **51**(3), 242-9.

[271] Strobl, H., Knapp, W. (1999) TGFβ 1 regulation of dendritic cells. *Microbes & Infection,* **1**(15), 1283-90.

[272] Van Vlasselaer, P., Punnonen, J., de Vries, J.E. (1992) Transforming growth factor-beta directs IgA switching in human B cells. *Journal of Immunology*, **148**(7), 2062-7.

[273] Cazac, B.B., Roes, J. (2000) TGFβ receptor controls B cell responsiveness and Induction of IgA *in vivo*. *Immunity*, **13**, 443-451.

[274] Elgert, K.D., Alleva, D.G., Mullins, D.W. (1998) Tumor-induced immune dysfunction: the macrophage connection. *J. Leukoc. Biol.*, **64**(3), 275-290.

[275] Reed, S.G. (1999) TGFβ in infections and infectious disease. *Microbes & Infection,* **1**(15), 1315-25.

[276] Li, X.F., Takiuchi, H., Zou, J.P., Katagiri, T., Yamamoto, N., Nagata, T., Ono, S., Hamaoka, T. (1993) Transforming growth factor-beta (TGFβ)-mediated immunosuppression in the tumor-bearing state: enhanced production of TGFβ and a progressive increase in TGFβ susceptibility of anti-tumor CD4+ T cell function. *Jpn. J. Cancer Res.*, **84**, 315-25.

[277] De Visser, K.E., Kast, W.M. (1999) Effects of TGFβ on the immune system: implications for cancer immunotherapy. *Leukemia,* **13**, 1188-99.

[278] Hsieh, C.L., Chen, D.S., Hwang, L.H. (2000) Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. *Human Gene Therapy,* **11**, 681-92.

[279] Lahn, M., Fisch, P., Kohler, G., Kunzmann, R., Hentrich, I., Jesuiter, H., Behringer, D., Muschal, B., Veelken, H., Kulmburg, P., Ikle, D.N., Lindemann, A. (1999) Pro-inflammatory and T cell inhibitory cytokines are secreted at high levels in tumor cell cultures of human renal cell carcinoma. *Eur. Urol.*, **35**(1), 70-80

[280] Vanky, F., Nagy, N., Hising, C., Sjovall, K., Larson, B., Klein, E. (1997) Human *ex vivo* carcinoma cells produce transforming growth factor beta and thereby can inhibit lymphocyte functions *in vitro*. *Cancer Immunol. Immunotherapy,* **43**(6), 317-23.

[281] Pollack, J.R., Perou, C.M., Alizadeh, A.A., Eisen, M.B., Pergamenschikov, A., Williams, C.F., Jeffrey, S.S., Botstein, D., Brown, P.O. (1999) Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat. Genet.*, **23**, 41-46.

[282] Pandey, A., Mann, M. (2000) Proteomics to study genes and genomes. *Nature*, **405**, 837-846.

[283] Velculescu, V.E., Zhang, L., Vogelstein, B., Kinzler, K.W. (1995) Serial analysis of gene expression. *Science*, **270**, 484-487.

[284] Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N., Doetschman, T. (1992) Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature*, **359**(6397), 693-9.

[285] Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., Karlsson, S. (1993) Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA*, **90**(2), 770-4.

[286] Boivin, G.P., O'Toole, B.A., Orsmby, I.E., Diebold, R.J., Eis, M.J., Doetschman, T., Kier, A.B. (1995) Onset and progression of pathological lesions in transforming growth factor-beta 1-deficient mice. *Am. J. Pathol.*, **146**(1), 276-88.

[287] Kulkarni, A.B., Ward, J.M., Yaswen, L., Mackall, C.L., Bauer, S.R., Huh, C.G., Gress, R.E., Karlsson, S. (1995) Transforming growth factor-beta 1 null mice. An animal model for inflammatory disorders. *Am. J. Pathol.*, **146**(1), 264-75.

[288] Christ, M., McCartney-Francis, N.L., Kulkarni, A.B., Ward, J.M., Mizel, D.E., Mackall, C.L., Gress, R.E., Hines, K.L., Tian, H., Karlsson, S. *et al.* (1994) Immune dysregulation in TGFβ 1-deficient mice. *J. Immunol.*, **153**(5), 1936-46.

[289] Tang, B., Bottinger, E.P., Jakowlew, S.B., Bagnall, K.M., Mariano, J., Anver, M.R., Letterio, J.J., Wakefield, L.M. (1998) Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat. Med.* **4**(7), 802-7.

[290] Engle, S.J., Hoying, J.B., Boivin, G.P., Ormsby, I., Gartside, P.S., Doetschman, T. (1999) Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res.*, **59**(14), 3379-86.

[291] Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., Boivin, G.P., Cardell, E.L., Doetschman, T. (1997) TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development*, **124**(13), 2659-70.

[292] Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., Ferguson, M.W., Doetschman, T. (1995) Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat. Genet.*, **11**(4), 409-14.

[293] Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., Groffen, J. (1995) Abnormal lung development and cleft palate in mice lacking TGFβ 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.*, **11**(4), 415-21.

[294] Oshima, M., Oshima, H., Taketo, M.M. (1996) TGFβ receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev. Biol.*, **179**, 297-302.

[295] Waldrip, W.R., Bikoff, E.K., Hoodless, P.A., Wrana, J.L., Robertson, E.J. (1998) Smad2 signaling in extraembryonic

tissues determines anterior-posterior polarity of the early mouse embryo. *Cell*, **92**(6), 797-808.

[296] Nomura, M., Li, E. (1998) Smad2 role in mesoderm formation, left-right patterning and craniofacial development. *Nature*, **393**(6687), 786-90.p

[297] Weinstein, M., Yang, X., Li, C., Xu, X., Gotay, J., Deng, C.X. (1998) Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppressor smad2. *Proc. Natl. Acad. Sci. USA*, **95**(16), 9378-83.

[298] Heyer, J., Escalante-Alcalde, D., Lia, M., Boettinger, E., Edelmann, W., Stewart, C.L., Kucherlapati, R. (1999) Postgastrulation Smad2-deficient embryos show defects in embryo turning and anterior morphogenesis. *Proc. Natl. Acad. Sci. USA*, **96**(22), 12595-600.

[299] Datto, M.B., Frederick, J.P., Pan, L., Borton, A.J., Zhuang, Y., Wang, X.F. (1999) Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol. Cell Biol.*, **19**(4), 2495-504.

[300] Zhu, Y., Richardson, J.A., Parada, L.F., Graff, J.M. (1998) Smad3 mutant mice develop metastatic colorectal cancer. *Cell*, **94**(6), 703-14.

[301] Yang, X., Letterio, J.J., Lechleider, R.J., Chen, L., Hayman, R., Gu, H., Roberts, A.B., Deng, C. (1999) Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGFβ *EMBO. J.*, **18**(5), 1280-91.

[302] Sirard, C., de la Pompa, J.L., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L., Kern, S.E., Rossant, J., Mak, T.W. (1998) The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev.*, **12**(1), 107-19.

[303] Yang, X., Xu, X., Li, C., Deng, C.X. (1998) The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. *Proc. Natl. Acad. Sci*, **95**, 3667-72.

[304] Taketo, M.M., Takaku, K. (2000) Gastro-intestinal tumorigenesis in Smad4 mutant mice. *Cytokine Growth Factor Rev.*, **11**(1-2), 147-57.

[305] Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M.F., Taketo, M.M. (1998) Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell*, **92**, 645-656.

[306] Yang, X., Castilla, L.H., Xu, X., Li, C., Gotay, J., Weinstein, M., Liu, P.P., Deng, C.X. (1999) Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development*, **126**(8), 1571-80.

[307] Chang, H., Zwijsen, A., Vogel, H., Huylebroeck, D., Matzuk, M.M. (2000) Smad5 is essential for left-right asymmetry in mice. *Dev. Biol.*, **219**(1), 71-8.

[308] Chang, H., Huylebroeck, D., Verschueren, K., Guo, Q., Matzuk, M.M., Zwijsen, A. (1999) Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development*, **126**(8), 1631-42.

[309] Galvin, K.M., Donovan, M.J., Lynch, C.A., Meyer, R.I., Paul, R.J., Lorenz, J.N., Fairchild-Huntress, V., Dixon, K.L., Dunmore, J.H., Gimbrone, M.A. Jr., Falb, D., Huszar, D. (2000) A role for smad6 in development and homeostasis of the cardiovascular system. *Nat. Genet.*, **24**, 171-4.

[310] Esmatjes, E., Flores, L., Lario, S., Claria, J., Cases, A. (1999) Smoking increases serum levels of Transforming growth factor-β in diabetic patients. *Diabetes Care*, **22**, 1915-1916.

[311] Iglesias-De La Cruz, M.C., Ruiz-Torres, P., Alcami, J., Diez-Marques, L., Ortega-Velazquez, R., Chen, S., Rodriguez-Puyol, M., Ziyadeh, F., Rodriguez-Puyol, D. (2001) Hydrogen peroxide increases extracellular matrix mRNA through TGFβ in human mesangial cells. *Kidney Int.*, **59**, 87-95.

* The induction of TGFβ by oxidative stress, which in turn causes expression of ECM components, may underlie the development and progression of certain nephropathies.

[312] Thiagalingam, S., Laken, S., Willson, J.K., Markowitz, S.D., Kinzler, K.W., Vogelstein, B., Lengauer, C. (2001) Mechanisms underlying losses of heterozygosity in human colorectal cancers. *Proc. Natl. Acad. Sci. USA*, **98**(5), 2698-2702.

[313] Zhou, W., Galizia, G., Goodman, S.N., Romans, K.E., Kinzler, K.W., Vogelstein, B., Choti, M.A., Montgomery, E.A. (2001) Counting alleles reveals a connection between chromosome 18q loss and vascular invasion. *Nat. Biotechnol.*, **19**, 78-81.

[314] Fink, S.P., Swinler, S.E., Lutterbaugh, J.D., Massague, J., Thiagalingam, S., Kinzler, K.W., Vogelstein, B., Willson, J.K., Markowitz, S. (2001) Transforming growth factor-beta-induced growth inhibition in a Smad4 mutant colon adenoma cell line. *Cancer Res.*, **61**(1), 256-60.

** TGFβ signaling can operate in a Smad4 independent pathway.

[315] Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., Gocayne, J.D., Amanatides, P., Ballew, R.M., Huson, D.H., Wortman, J.R., Zhang, Q., Kodira, C.D., Zheng, X.H., Chen, L., Skupski, M., Subramanian, G., Thomas, P.D., Zhang, J., Gabor, Miklos, G.L., Nelson, C., Broder, S., Clark, A.G., Nadeau, J., McKusick, V.A., Zinder, N., Levine, A.J., Roberts, R.J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di, Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A.E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T.J., Higgins, M.E., Ji, R.R., Ke, Z., Ketchum, K.A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G.V., Milshina, N., Moore, H.M., Naik, A.K., Narayan, V.A., Neelam, B., Nusskern, D., Rusch, D.B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M.L., Curry,

L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y.H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E.,Thomas, R., Tint, N.N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J.F., Guigo, R., Campbell, M.J., Sjolander, K.V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T.,Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y.H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A., Zhu, X. (2001) The Sequence of the Human Genome. *Science*, **291**, 1304-1351.

[316] Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., Lancet, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.P., Yeh, R.F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Patrinos, A., Morgan, M.J., Szustakowki, J., de Jong, P., Catanese, J.J., Osoegawa, K., Shizuya, H., Choi, S., Chen, Y.J. (2001) Initial sequencing and analysis of the human genome. International Human Genome Sequencing Consortium. *Nature*, **409**, 860-921.

Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence

Sam Thiagalingam, PhD,*† Rebecca L. Foy, BS,* Kuang-hung Cheng, MS,† Hyunjoo J. Lee, BA,* Arunthathi Thiagalingam, PhD,‡ and Jose F. Ponte, PhD*

High frequency of chromosomal deletions elicited as losses of heterozygosity is a hallmark of genomic instability in cancer. Functional losses of tumor suppressor genes caused by loss of heterozygosity at defined regions during clonal selection for growth advantage define the minimally lost regions as their likely locations on chromosomes. Loss of heterozygosity is elicited at the molecular or cytogenetic level as a deletion, a gene conversion, single or double homologous and nonhomologous mitotic recombinations, a translocation, chromosome breakage and loss, chromosomal fusion or telomeric end-to-end fusions, or whole chromosome loss with or without accompanying duplication of the retained chromosome. Because of the high level of specificity, loss of heterozygosity has recently become invaluable as a marker for diagnosis and prognosis of cancer. The molecular defects for the occurrence of loss of heterozygosity are derived from disabled caretaker genes, which protect the integrity of DNA, or chromosome segregator genes, which mediate faithful chromosome disjunction. Curr Opin Oncol 2002, 14:65–72

*Genetics & Molecular Medicine Programs and Pulmonary Center, Department of Medicine and †Department of Pathology & Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts, USA; and ‡Bayer Corporation, East Walpole, Massachusetts, USA.

Correspondence to Sam Thiagalingam, PhD, Genetics Program, Boston University School of Medicine, 715 Albany Street, E603, Boston, MA 02118, USA; e-mail: samthia@bu.edu

Current Opinion in Oncology 2002, 14:65–72

Abbreviations

DSB	double-strand break
HEJ	homologous end joining
LOH	loss of heterozygosity
MLR	minimally lost region
NHEJ	nonhomologous end joining
TSG	tumor suppressor gene

ISSN 1040–8746

Genetic alterations and cancer

Human cancer is generally thought to develop as a result of loss of or irreversible damage to critical genes in a multistep process involving the accumulation of genetic alterations. More than 100 years ago, Theodor Boveri wrote a remarkable book, *The Origins of Malignant Tumors* [1,2], in which he suggested chromosome missegregation leading to aneuploidy, an abnormal balance of chromosomes, as the fundamental basis of cancer. The aneuploidy hypothesis has since been abandoned in favor of the gene mutation hypothesis [3,4••]. However, selection of nonparental aggressive karyotypic variants of cancer cells caused by genetic instability and mutations could be unified in aneuploidy to explain tumorigenesis [5–7,8••].

Overall, the genesis of cancer could be defined as the manifestation of loss of or abnormal function of genes affecting processes that maintain or regulate orderly normal cell function by both genetic and epigenetic mechanisms. The genetic basis of these functional anomalies could be derived from targeted aberrations in the regulatory elements or functional domains because of mutations or loss caused by deletions of small or large contiguous genetic material affecting nonessential but critical genes required for normal differentiated function. During localized evolution, tumor cells aggressively proliferate and invade and spread to distant sites. At the same time, the genetic material could undergo accompanying changes, potentially in small increments, to aid in clonal evolution, increasing the genetic heterogeneity of the tumor cells. Therefore, the genetic outlook and the biochemical properties of the tumor cells at the time of initiation, could be entirely different from their characteristics at an advanced stage of cancer. These abnormalities in the blueprint of the genetic material could be derived from defective oncogenes, which become activated because of alteration of one allele, or from tumor suppressor genes (TSGs), whose functionality is eliminated when both alleles are damaged or lost.

Loss of heterozygosity and mapping of tumor suppressor genes in cancer

A statistical study of a childhood cancer, retinoblastoma, led to the proposal of the two-mutation hypothesis for the initiation of cancer, in which the first mutation could

be either germinal or somatic and the second was always somatic [9,10,11••]. This hypothesis was experimentally confirmed in subsequent studies by the demonstration of loss of heterozygosity (LOH) at the *RB1* locus in retinoblastoma patients carrying a germ line mutation of the *RB1* gene [12,13]. LOH, defined as a loss of one allele at a constitutional (germline) heterozygous locus, has been accepted as a hallmark of one of the two hits required for the inactivation of TSGs in cancer.

Loss of heterozygosity analyses of solid tumors have not only enabled the delineation of specific minimally lost regions (MLRs) as the likely locations of critical TSGs but also provided the molecular portrait of the pattern of accumulation of genetic alterations in a multistep progression of cancer [14–18,19••]. Confirmed TSGs could have been isolated by either linkage studies or LOH analyses of sporadic tumors, because the MLRs almost always point to the map position of critical TSGs involved in different types of cancers [19••,20–30,31•]. A TSG involved in a sporadic cancer could be a familial gene for a different cancer, and *vice versa* (Table 1).

A plethora of evidence supporting sites of recurrent LOH, which underlies the growth advantage required for tumorigenesis, still has not resolved the question of whether the mutation or LOH occurs first to initiate cancer. Localized double deletions (homozygous) targeting a specific gene or locus are rare. LOH patterns and the extent of each deletion in clonally selected tumor cells are highly specific to the chromosome [19••]. These discrepancies apparently reflect the size of the chromosome (*ie*, smaller chromosomes exhibit whole chromosome losses more frequently, whereas larger ones predominantly harbor partial losses) and selective pressure imposed by the remaining genes, which are essential for cell survival or provide a growth advantage during clonal selection of cells that eventually form the tumor. Overall, when one of the hits is LOH encompassing the entire chromosome, a portion, or a localized region, the second hit is inactivation of the actual target gene caused by a second reciprocal LOH event (homozygous), a mutation, or an epigenetic alteration resulting in loss of gene function.

Despite the confirmation of two-hit inactivation of TSGs in several cancers, the mode of inactivation itself may vary in a gene-specific or tumor-specific manner. Two of the most historically celebrated TSGs are *Rb1* and *p53*. Retinoblastoma and its associated tumors, such as osteosarcoma, were found to harbor consistently either a homozygous deletion of the *Rb1* locus at 13q14 or a mutation of the remaining allele in a tumor with LOH [17]. A consistent region of LOH at 17p13, observed initially in colon cancer, led to the rediscovery of the *p53* gene as a TSG [32,33]. Missense mutations were the primary mechanism of inactivation of the remaining allele, and *p53* was later confirmed as the most frequent target for inactivation in a variety of cancers [32–34]. These observations are consistent with mutational inactivation of the target gene followed by LOH in familial cancers (*eg*, retinoblastoma, Li-Fraumeni) as the predominant mechanism caused by a predisposing genetic alteration, whereas in sporadic cancers, the mode of inactivation of the target gene may be variable, but the ultimate outcome is the loss of function. Exceptions to these rules have been observed in several cancers, and whether mutation or LOH occurs first in target inactivation is being debated [35••,36••,37•]. Although this debate is primarily based on studies of sporadic cancers in which a normal or mutant allele of the suspected target TSG has been observed in tumors exhibiting LOH, the explanation of these assessments could be more complicated because of the potential presence of multiple target genes, alternate modes of inactivation such as epigenetic silencing of gene expression by promoter methylation, or linkage disequilibrium with the true target gene.

The inactivation of the *Smad2* and *Smad4* genes, localized to the MLR at 18q21 in colorectal cancer, is consistent with the multiple target gene theory, in which true inactivation of both genes has been demonstrated [25,38]. The *p16* (*CDKN2A*) gene localizes to chromosome 9p21, also a hot spot for inactivation by LOH. Al-

Table 1. Tumor suppressor genes and cancers

Gene	Map position	Familial cancer syndromes	Associated sporadic cancers
APC	5q21	Adenomatous polyposis coli and Turcot syndrome	Colon and brain cancers
BRCA1	17q21	Breast/ovarian cancer	Breast, ovarian, and prostate cancer
BRCA2	13q12–13	Breast cancer	Breast, ovarian, and pancreatic cancers
NF1	17q11.2	Neurofibromatosis type I	Neurofibromatosis, colon carcinoma, and astrocytoma
NF2	22q12.2	Neurofibromatosis type II	Vestibular schannoma, meningioma, and ependymoma
$p16^{MTS1}$	9p21	Melanoma	Glioblastoma, melanoma, and cancers of the pancreas, breast, and other organs
p53	17p13	Li-Fraumeni syndrome	50% of all cancers, including breast, brain, lung, colon, bladder, ovarian, and prostate
RB1	13q14.3	Retinoblastoma	Retinoblastoma, osteosarcoma, bladder carcinoma, and cancers of the breast and lung
SMAD4	18q21	Juvenile polyposis	Pancreatic, colon, and lung cancers
PTEN	10q24–25	Cowden disease	Brain, breast, and prostate cancers
VHL	3p25–26	von Hippel-Lindau syndrome	Renal cell carcinoma and pheochromocytoma
WT1	11p13–15	Wilm tumor	Nephroblastoma

terations in *p16* occur frequently in lung, skin, and most other common forms of human cancer [39,40]. In many tumor types, point mutations in the *p16* gene are rare, and LOH of 9p21 is not always observed in tumors. However, there is increasing evidence for loss of *p16* gene function via transcriptional silencing associated with abnormal DNA methylation of the transcription regulatory region [41]. Allele-specific methylation at particular sites may be somatic or may occur in the germ line, affecting all cells. Therefore, the two-hit hypothesis of TSG inactivation by Knudson [9–11] could be extended to explain that the first hit, the second hit, or both could result from methylation, leading to complete inactivation of the gene [42,43••]. The exploitation of the known modes of inactivation of the TSGs to tumors harboring LOH of a specific region may not be sufficient to account completely for the two hits required for the inactivation in all tumors. This situation has been frequently encountered with several TSGs, including *p16* and *PTEN*, during the analyses of melanomas and glioblastomas, and even with *p53* in some cases of Li-Fraumeni, implying that these suggested target genes may be in linkage disequilibrium with the true target gene or may be inactivated at a different point of the functional pathway controlled by these genes [44,45••, 46,47••,48,49]. Delineation of all candidate genes localized to the MLRs and painstaking evaluations to determine their loss of function unambiguously would be necessary to answer these questions. Assignment of true target genes is a tedious process but could be expedited by technologic advances in analyzing loss of function of genes and improvements in making accurate genome sequence data available in a short time using a high throughput method.

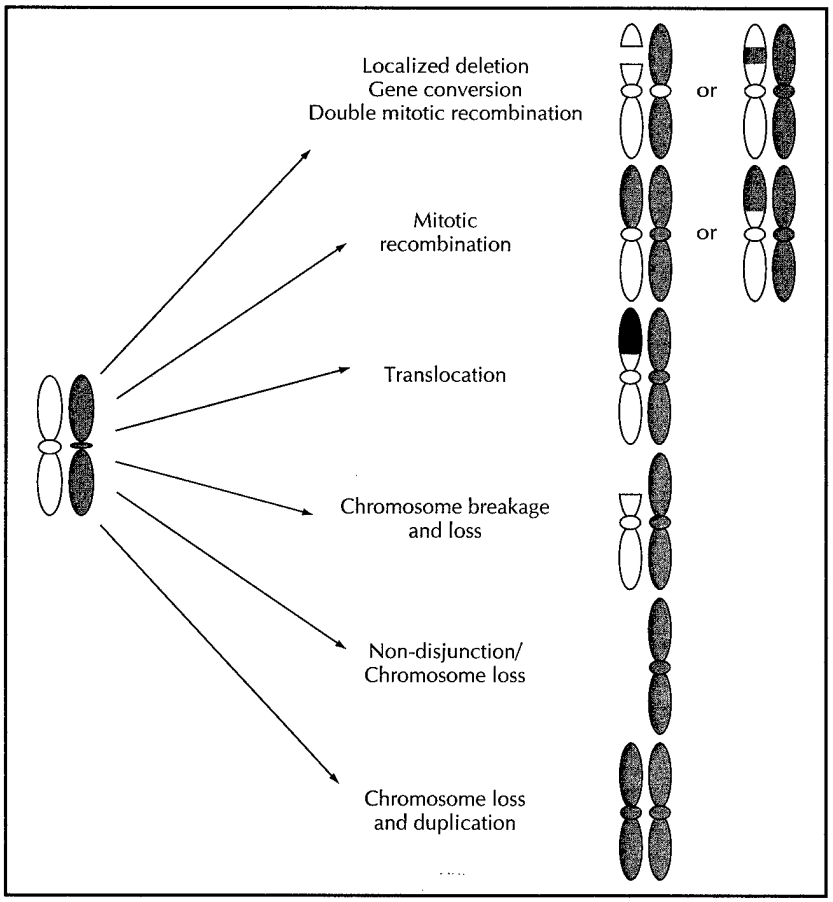
Identification of target genes is actively pursued on one front, but utilization of available LOH data as markers for diagnosis and prognosis of cancer also has become generally accepted. A higher frequency of consistent LOH at defined chromosomal regions critical for specific cancers has made this a useful, reliable DNA marker for diagnosis and prognosis of cancer, regardless of whether the target gene has been identified [50–53].

Molecular elicitation of loss of heterozygosity

A heuristic model summarizing the molecular elicitation of LOH in a variety of tumors is presented in Figure 1. Variations in the patterns in which LOH could occur include the following:

Figure 1. A model for molecular elicitation of loss of heterozygosity

Events such as localized deletions with accompanying chromatin loss resulting from one or two double-strand DNA breaks, gene conversion, single or double mitotic recombination, translocation, chromosomal nondisjunction, and chromosomal nondisjunction accompanied by duplication of the remaining chromosome could lead to loss of heterozygosity (LOH). The loss of genes localized to the regions of LOH could result in haploinsufficiency or unmasking of the functional expression of a recessive or deficient allele.



(1) Localized loss of one allele or gene in a highly specific manner could be accomplished by a simple deletion resulting from two double-strand breaks or double mitotic recombination involving the homologous chromosomal arm. When this occurs, the unaffected genetic material remains contiguous. If the loss involved a specific gene, it can be regarded as a gene conversion. Allele-specific or gene-specific probes and flanking probes would be necessary to detect such alterations. Although these microdeletions are difficult and laborious to find because of the enormous amount of effort required to analyze numerous tumor samples, at least two recent reports, one analyzing the *NF1* locus on 17q in neurofibromas and the other delineating a highly specific localized LOH on chromosome 1p for colon cancers, substantiate the claim that interstitial deletions targeting a single gene or allele could occur [19••,54••]. Additionally, highly specific, localized homozygous deletions observed within a gene or locus also indicate that a double hit could occur simply by targeted loss of genetic material [25,55–57].

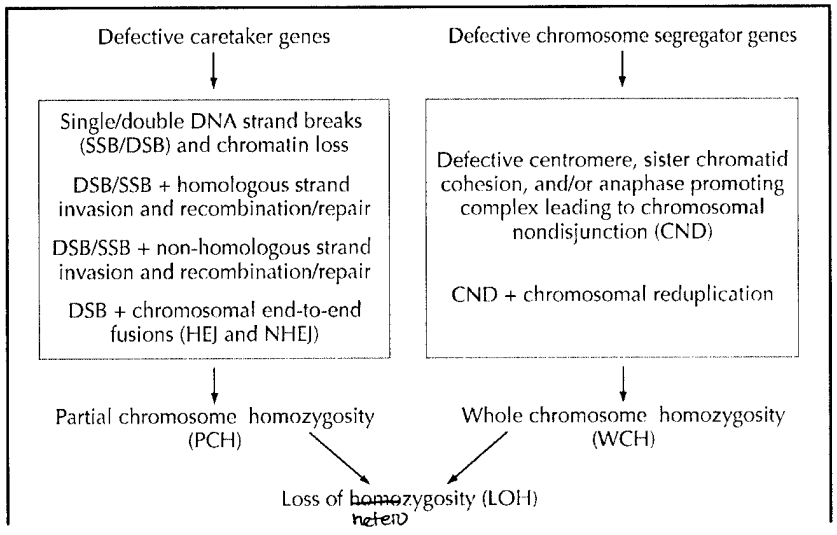
(2) Extensive loss of genetic material involving a portion of or an entire chromosomal arm could be accomplished by a double-strand break with the loss of genetic material distal to the break, a single mitotic recombination involving the homologous pair of chromosomes, or reciprocal or nonreciprocal translocation. Mitotic recombination is the result of a reciprocal exchange of genetic material between nonsister chromatids of homologous or nonhomologous chromosomes in mitotic cells as detected by substitution of contiguous markers with reference to an established marker. Although these are the most common genetic abnormalities described for tumor cells, in a recent landmark study, the authors confirmed the previous observations and suggestions and provided comparative and direct molecular genetic and cytogenetic evidence from the same tumor-derived cells for the existence of isochromosomes, translocations, and complete loss of genetic material to support additional mechanisms for LOH [12,14,19••,25,30,57–61].

(3) Loss of a whole chromosome is generally accomplished by nondisjunction defects in chromosome segregation [26,32]. However, the existence of multiple copies of chromosomes in tumor cells deemed to have lost one member of the homologous pair by cytogenetic analyses confirms reduplication of the remaining chromosome leading to homozygosity [19••,59,62,63].

Roles of caretaker and chromosome segregator genes in loss of heterozygosity

Multiple interconnected mechanisms have evolved to ensure the maintenance and faithful partitioning of genetic material at cell division. The failure of cellular functions to maintain the genetic integrity of the genome by faithful DNA replication, DNA damage repair, telomere protection, segregation of chromosomes at mitosis, or unscheduled recombination could lead to genomic instability, which is elicited as LOH. The two major categories of chromosomal abnormalities that involve portions of a chromosome or an entire chromosome could be considered derived from defects in caretaker genes or chromosome segregator genes, respectively (Fig. 2). Broadly, a caretaker gene is any gene required to maintain the integrity of DNA during processes such as DNA replication, repair, or recombination; telomere maintenance and protection; and chromosome packaging; or, to protect the DNA from nucleases and other adversities of intracellular physiologic byproducts [64–66,67•,68•,69••]. The chromosome segregator genes are those required to mediate orderly disjunction of sister chromatids to the daughter cells during a mitotic cell division. These genes could include all those involved in determining the cen-

Figure 2. A model for the genetic and molecular mechanistic basis of loss of heterozygosity



Loss of heterozygosity (LOH) occurs because of defective caretaker genes, defective chromosome segregator genes, or both. Defects in both could promote LOH simultaneously in tumor cells affecting the same or different chromosomes. Defective caretaker genes are involved primarily in exchange or loss of the entire chromosome arm or a portion, and defective segregator genes are involved primarily in whole chromosome loss. Deletions of portions of a chromosome result in partial chromosomal homozygosity (PCH), whereas loss of entire chromosome with or without accompanying duplication of the remaining chromosome causes whole chromosome homozygosity (WCH).

tromere structure, sister chromatid cohesion, and the anaphase promoting complex, and others required for the proper assembly of the bipolar spindle apparatus to ensure faithful segregation of genetic information [70,71, 72••,73,74•].

Single-strand DNA interruptions, breaks, or gaps could arise during DNA damage from a variety of causes, such as exposure to ultraviolet radiation, hydrogen peroxide or alkylating agents, DNA replication accompanied by processes such as defective nucleotide excision repair or mismatch repair, or defective DNA replication resulting in the accumulation of the lagging strand caused by defective DNA ligase [65,75–78]. Single-strand DNA generated during these events could enhance mitotic recombination and lead to an exchange of genetic material from homologous or nonhomologous chromosomes. A number of genetically determined disorders are known to cause susceptibility to chromosomal breaks, cause increased frequency of breaks, and promote interchanges that occur either spontaneously or after exposure to various DNA-damaging agents [79••].

The DNA lesions resulting in double-strand breaks (DSBs) are among the most fatal, because they disrupt the continuity of the DNA template essential for DNA replication and transcription. A broken chromatid acquiring a new telomere leads to the loss of DNA distal to the break, resulting in LOH. DSBs could arise because of stalling of the replication fork during DNA synthesis; defective DNA repair; DNA damaging effects such as ionizing radiation; cleavage by specific enzymes such as V(D)J recombinase; or recurrent chromosomal breaks at susceptible DNA sequences caused by tandem repeat DNA instability at microsatellite (CCG)n, AT-rich minisatellites, other minisatellites, or defined or unique signal sequences [80–84]. DSBs are highly recombinogenic and represent a major threat to the integrity of the genome. Although most DSBs are rejoined through repair pathways known as nonhomologous end joining (NHEJ) or homologous end joining (HEJ) pathways, they could also initiate homologous or non homologous strand invasion and recombination and repair [78,81,85,86••,87]. Mutations in the genes involved in these pathways display dramatic genomic instability ranging from chromosomal fragmentation to nonreciprocal translocations [88••,89,90••]. Homologous and nonhomologous recombination and repair, HEJ, and NHEJ involving homologous chromosomes and nonhomologous chromosomes would lead to partial chromosome homozygosity resulting in LOH (Fig. 2). The NHEJ and HEJ pathways would create translocations and isochromosomes, which could be detected cytogenetically using chromosome painting methods [19••].

Telomeres are specialized nucleoprotein complexes that serve to protect the ends of linear chromosomes from recombination, fusion, and recognition as damaged DNA. Lack of functional telomeres in rapidly dividing cancer cells could occur because of defects in or low levels of telomerase or telomere binding proteins such as TRF1 and TRF2 and cause chromosome end-to-end (telomeric) fusion. The fusion-bridge-breakage process of chromosomes fused at their telomeres during chromosomal disjunction could initiate a wide array of chromosomal aberrations caused by the generation of DSBs, which could, in turn, cause a chain of events such as DNA strand invasions and chromosomal fusions [91•,92,93••]. If telomere attrition caused by loss of capping activity and severe genomic instability continues in cancer cells, it may lead to crisis and cell death [91•,93••]. Therefore, in most human cancers, telomerase usually becomes activated at the time of transition to advanced invasive cancer, enabling stable inheritance of these genetic alterations to the progeny cells [91•,92,93••]. The telomeric end-to-end fusions resulting in metacentric chromosomes and translocations involving portions of chromosomes also leads to partial chromosome homozygosity [19••] (Fig. 2).

Despite LOH analysis indicating loss of one of the chromosomes of the homologous pair caused by reduction to homozygosity based on all the markers analyzed, cytogenetic analysis by chromosome painting has revealed the existence of multiple copies of the chromosome being investigated [19••]. These results are consistent with defects in chromosome segregator genes coupled with duplication of the remaining chromosome, leading to whole chromosome homozygosity resulting in LOH (Fig. 2). The defects in chromosome segregator genes that are required for faithful disjunction of sister chromatids to the daughter cells could comprise genes that (1) participate in forming the centromere structure, (2) associate with the centromere during the metaphase to anaphase transition, (3) mediate the sister chromatid cohesion, and (4) play a role in the anaphase-promoting complex, including the proper assembly of the bipolar spindle apparatus, to ensure faithful segregation of genetic information [70,71,72••,73,74•,94,95,96••,97,98, 99••]. However, study of these genes to determine the nodal targets most frequently affected in cancers is still in its infancy. Although Cahill *et al.* [100••] demonstrated inactivation of *hBUB1* and *hBUBR1* genes in colorectal cancers exhibiting chromosomal instability, subsequent progress has been slow, perhaps because of the large number of target genes that could become inactivated or the epigenetic mechanisms of inactivation of these genes [101,102,103••]

Conclusions

Despite an enormous amount of genomic sequence and contig data becoming available from the human genome project, the major challenge is to find the disease-causing genes that will make an impact on overall health care and

management. The exploitation of LOH analyses using traditional and high throughput methods such as bacterial artificial chromosome microarrays to define MLRs provides a distinct advantage to cancer geneticists in discovering target TSGs in this era of genomics. The LOH studies have not only provided a view of the genetic abnormalities of complex diseases such as cancer but also stimulated the drive to obtain a better understanding of the various cellular processes such as DNA repair, replication, recombination, telomere maintenance, and cell division by providing excellent, highly discernible visual examples resulting from their defects.

Acknowledgments

Work in the authors' laboratory is supported by grants from the American Lung Association (RG-013-N), the American Cancer Society (72-001-26-IRG), the National Institutes of Health (RO1 ES10377), the Evans Medical Foundation, the Dolphin Trust Investigator Award from the Medical Foundation, and the Career Development Award from the Department of Defense (DAMD 17-01-1-0160). R.L.F., K.C., and J.F.P. are supported by the American Lung Association, the Medical Foundation, and a National Institutes of Health training grant (T32HL07035).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • Of special interest
- •• Of outstanding interest

1 Boveri TH: Uber die Natur der centrosomen, Zellen-Studien G IV, Edited by Fischer; 1900.

2 Boveri TH: Zur Frage der Enstehung maligner Tumoren, Zellen-Studien G IV, Edited by Fischer Verlag. Baltimore: Williams and Wilkins; 1914.

3 Boland CR, Ricciardiello L: How many mutations does it take to make a tumor? Proc Natl Acad Sci U S A 1999, 96:14675–14677.

4 •• Loeb KR, Loeb LA: Significance of multiple mutations in cancer. Carcinogenesis 2000, 21:379–385.
Tumor progression is discussed as the selection of cells with specific mutations in genes that normally function in maintaining genetic stability.

5 Lengauer C, Kinzler KW, Vogelstein B: Genetic instabilities in human cancers. Nature 1998, 396:643–649.

6 Duesberg P, Rasnick D, Li R, et al.: How aneuploidy may cause cancer and genetic instability. Anticancer Res 1999, 19:4887–4906.

7 Duesberg P: Are centrosomes or aneuploidy the key to cancer? Science 1999, 284:2091–2092.

8 •• Li R, Sonik A, Stindl R, et al.: Aneuploidy vs. gene mutation hypothesis of cancer: recent study claims mutation but is found to support aneuploidy. Proc Natl Acad Sci U S A 2000, 97:3236–3241.
Evidence is provided for aneuploidy-initiated karyotypic evolution that could generate clones of tumorigenic cells.

9 Knudson AG: Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 1971, 68:820–823.

10 Knudson AG: Genetics of human cancer. Genetics 1975, 79(suppl):305–316.

11 •• Knudson AG: Chasing the cancer demon. Annu Rev Genet 2000, 34:1–19.
In this review, Knudson describes the historical prospective of the discovery of TSGs.

12 Cavenee WK, Dryja TP, Phillips RA, et al.: Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 1983, 305:779–784.

13 Godbout R, Dryja TP, Squire J, et al.: Somatic inactivation of genes on chromosome 13 is a common event in retinoblastoma. Nature 1983, 304:451–453.

14 Vogelstein B, Fearon ER, Hamilton SR, et al.: Genetic alterations during colorectal-tumor development. N Engl J Med 1988, 319:525–532.

15 Vogelstein B, Fearon ER, Kern SE, et al.: Allelotype of colorectal carcinomas. Science 1989, 244:207–211.

16 Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990, 61:759–767.

17 Lasko D, Cavenee W, Nordenskjold M: Loss of constitutional heterozygosity in human cancer. Annu Rev Genet 1991, 25:281–314.

18 Yokota J, Sugimura T: Multiple steps in carcinogenesis involving alterations of multiple tumor suppressor genes. FASEB J 1993, 7:920–925.

19 •• Thiagalingam S, Laken S, Willson JKV, et al.: The mechanisms underlying losses of heterozygosity in human colorectal cancers. Proc Natl Acad Sci U S A 2001, 98:2698–2702.
A detailed account of the various modes of elicitation of LOH is presented with the help of molecular genetic and cytogenetic data generated using highly homogenous tumor material from colon cancer.

20 Karp JE, Broder S: Molecular foundations of cancer: new targets for intervention. Nat Med 1995, 1:309–320.

21 Williams BO, Jacks T: Mechanisms of carcinogenesis and the mutant mouse. Curr Opin Genet Dev 1996, 6:65–70.

22 Casey G, Lopez ME, Ramos JC, et al.: DNA sequence analysis of exons 2 through 11 and immuno-histochemical staining are required to detect all known p53 alterations in human malignancies. Oncogene 1996, 13:1971–1981.

23 Harris CC: p53 tumor suppressor gene: from the basic research laboratory to the clinic–an abridged historical perspective. Carcinogenesis 1996, 17:1187–1198.

24 Hahn SA, Hoque AT, Moskaluk CA, et al.: Homozygous deletion map at 18q21.1 in pancreatic cancer. Cancer Res 1996, 56:490–494.

25 Thiagalingam S, Lengauer C, Leach FS, et al.: Evaluation of candidate tumor suppressor genes on chromosome 18 in colorectal cancers. Nat Genet 1996, 13:343–346.

26 Schutte M, Hruban RH, Hedrick L, et al.: DPC4 gene in various tumor types. Cancer Res 1996, 56:2527–2530.

27 Uchida K, Nagatake M, Osada H, et al.: Somatic in vivo alterations of the JV18–1 gene at 18q21 in human lung cancers. Cancer Res 1996, 56:5583–5585.

28 Wooster R, Bignell G, Lancaster J, et al.: Identification of breast cancer susceptibility gene, BRCA2. Nature 1995, 378:789–792.

29 Li J, Yen C, Liaw D, et al.: PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast and prostate cancer. Science 1997, 275:1876–1878.

30 Virmani AK, Fong KM, Kodagoda D, et al.: Genes Chromosomes Cancer 1998, 21:308–319.

31 • Elo JP, Visakorpi T: Molecular genetics of prostate cancer. Ann Med 2001, 33:130–141.
A succinct report on prostate cancer genetics.

32 Baker SJ, Fearon ER, Nigro JM, et al.: Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 1989, 244:217–221.

33 Nigro JM, Baker SJ, Preisinger AC, et al.: Mutations in the p53 gene occur in diverse human tumor types. Nature 1989, 342:705–708.

34 Bennett WP, Hussain SP, Vahakangas, et al.: Molecular epidemiology of human cancer risk: gene-environment interactions and p53 mutation spectrum in human lung cancer. J Pathol 1999, 187:8–18.

35 •• Inoue H, Furukawa T, Sunamura M, et al.: Exclusion of SMAD4 mutation as an early genetic change in human pancreatic ductal tumorigenesis. Genes Chromosomes Cancer 2001, 31:295–299.
Because loss of 18q is an early event, long before any Smad4 mutations can be found in pancreatic ductal carcinomas, this report raises the possibility of other target genes.

36 •• Luttges J, Galehdari H, Brocker V, et al.: Allelic loss is often the first hit in the biallelic inactivation of the p53 and DPC4 genes during pancreatic carcinogenesis. Am J Pathol 2001, 158:1677–1683.
Protein expression and genetic data for *p53* and *DPC4* are presented as evidence for allelic loss as the first hit during biallelic inactivation of TSGs.

37 • Wilentz RE, Argani P, Hruban RH: Loss of heterozygosity or intragenic mutation, which comes first? Am J Pathol 2001, 158:1561–1563.
An analysis of the order of occurrence of LOH and mutation during inactivation of TSGs.

38 Riggins J, Thiagalingam S, Rozenblum E, et al.: MAD-related genes in the human. Nat Genet 1996, 13:347–349.

39 Kamb A, Gruis NA, Weaver J, et al.: A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994, 264:436–440.

40 Okamoto A, Hussain SP, Hagiwara K, et al.: Mutations in the $p16^{INK4/MST1/CDKN2}$, $p15^{INK4B/MST2}$, and p18 genes in primary and metastatic lung cancer. Cancer Res 1995, 55:1448–1451.

41 Merlo A, Herman JG, Mao L, et al.: 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor $p16^{INK4/MST1/CDKN2}$ in human cancers. Nat Med 1995, 1:686–692.

42 Jones PA, Laird PW: Cancer epigenetics comes of age. Nat Genet 1999, 21:163–167.

43 •• Baylin SB, Herman JG: DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 2000, 16:168–174.
This article provides insight into the role of DNA hypermethylation in TSG inactivation and its implications for use in diagnosis, prognosis, and therapy.

44 Liggett WH Jr, Sidransky D: Role of the p16 tumor suppressor gene in cancer. J Clin Oncol 1998, 16:1197–1206.

45 •• Harland M, Holland EA, Ghiorzo P, et al.: Mutation screening of the CDKN2A promoter in melanoma families. Genes Chromosomes Cancer 2000, 28:45–57.
Mutational analysis of 1 kb of the *CDKN2A* promoter region upstream of the start codon to identify causal mutations in melanoma families.

46 •• Mutter GL: PTEN, a protean tumor suppressor. Am J Pathol 2001, 158:1895–1898.
An excellent update on the *PTEN* gene as a target for inactivation in various cancers.

47 •• Snaddon J, Parkinson EK, Craft JA, et al.: Detection of functional PTEN lipid phosphatase protein and enzyme activity in squamous cell carcinomas of the head and neck, despite loss of heterozygosity at this locus. Br J Cancer 2001, 84:1630–1634.
Loss of heterozygosity at or close to the *PTEN* gene is frequently not associated with a mutation in the remaining allele.

48 Bell DW, Varley JM, Szydlo TE, et al.: Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 1999, 286:2528–2531.

49 Hirao A, Kong YY, Matsuoka S, et al.: DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science 2000, 287:1824–1827.

50 Sidransky D: Nucleic acid-based methods for the detection of cancer. Science 1997, 278:1054–1059.

51 Cairns P, Sidransky D: Molecular methods for the diagnosis of cancer. Biochim Biophys Acta 1999, 1423:C11–C18.

52 Nicholl ID, Dunlop MG: Molecular markers of prognosis in colorectal cancer. J Natl Cancer Inst 1999, 91:1267–1269.

53 Dong SM, Traverso G, Johnson C, et al.: Detecting colorectal cancer in stool with the use of multiple genetic targets. J Natl Cancer Inst 2001, 93:858–865.

54 •• Serra E, Rosenbaum T, Nadal M, et al.: Mitotic recombination effects homozygosity for NF1 germline mutations in neurofibromas. Nat Genet 2001, 28:294–296.
Evidence is presented to support mitotic recombination as a major mechanism for LOH at the *NF1* locus.

55 Friend SH, Bernards R, Rogelj S, et al.: A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 1986, 323:643–646.

56 Schutte M, da Costa LT, Hahn SA, et al.: Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region. Proc Natl Acad Sci U S A 1995, 92:5950–5954.

57 Hahn S, Schutte AM, Hoque AT, et al.: DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996, 271:350–353.

58 Cropp CS, Lidereau R, Campbell G, et al.: Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified. Proc Natl Acad Sci U S A 1990, 87:7737–7741.

59 Varella-Garcia M, Gemmill RM, Rabenhorst SH, et al.: Chromosomal duplication accompanies allelic loss in non-small cell lung carcinoma. Cancer Res 1998, 58:4701–4707.

60 Zhu X, Dunn JM, Goddard AD, et al.: Mechanisms of loss of heterozygosity in retinoblastoma. Cytogenet Cell Genet 1992, 59:248–252.

61 Bauer A, Savelyeva L, Claas A, et al.: Smallest region of overlapping deletion in 1p36 in human neuroblastoma: a 1 Mbp cosmid and PAC contig. Genes Chromosomes Cancer 2001, 31:228–239.

62 de Nooij-van Dalen AG, van Buuren-van Seggelen VH, Lohman PH, et al.: Chromosome loss with concomitant duplication and recombination both contribute most to loss of heterozygosity in vitro. Genes Chromosomes Cancer 1998, 21:30–38.

63 Knuutila S, Aalto Y, Autio K, et al.: DNA copy number losses in human neoplasms. Am J Pathol 1999, 155:683–694.

64 Kinzler KW, Vogelstein B: Cancer-susceptibility genes: gatekeepers and caretakers. Nature 1997, 386:761–763.

65 Yu Z, Chen J, Ford BN, et al.: Human DNA repair systems: an overview. Environ Mol Mutagen 1999, 33:3–20.

66 Heck MM: Condensins, cohesins, and chromosome architecture: how to make and break a mitotic chromosome. Cell 1997, 91:5–8.

67 • Kelly TJ, Brown GW: Regulation of chromosome replication. Annu Rev Biochem 2000, 69:829–880.
A detailed view on eukaryotic DNA replication, with descriptions of the players and regulators and the molecular basis of the process.

68 • Bishop AJ, Schiestl RH: Homologous recombination as a mechanism for genome rearrangements: environmental and genetic effects. Hum Mol Genet 2000, 9:2427–2434.
A good review on homologous recombination, with descriptions of the players and the mechanisms involved.

69 •• Artandi SE, DePinho RA: A critical role for telomeres in suppressing and facilitating carcinogenesis. Curr Opin Genet Dev 2000, 10:39–46.
An account of the role of telomeres in cancer, with an emphasis on *p53*-dependent and *p53*-independent effects.

70 Hirano T: SMC-mediated chromosome mechanics: a conserved scheme from bacteria to vertebrates? Genes Dev 1999, 13:11–19.

71 Nasmyth K, Peters JM, Uhlmann F: Splitting the chromosome: cutting the ties that bind sister chromatids. Science 2000, 288:1379–1385.

72 •• Saffery R, Irvine DV, Griffiths B, et al.: Nucleotide Human centromeres and neocentromeres show identical distribution patterns of >20 functionally important kinetochore-associated proteins. Hum Mol Genet 2000, 9:175–185.
A combination of immunofluorescence and fluorescent *in situ* hybridization methods were used to discern the various proteins involved in forming human centromeres and neocentromeres.

73 Tyler-Smith C, Floridia G: Many paths to the top of the mountain: diverse evolutionary solutions to centromere structure. Cell 2000, 102:5–8.

74 • Mitchison TJ, Salmon ED: Mitosis: a history of division. Nat Cell Biol 2001, 3: E17–E21.
A historical and mechanistic view of mitotic cell division is presented in this short review.

75 Lindahl T, Barnes DE: Mammalian DNA ligases. Annu Rev Biochem 1992, 61:251–281.

76 Grossman L, Thiagalingam S: Nucleotide excision repair, a tracking mechanism in search of damage. J Biol Chem 1993, 268:16871–16874.

77 Nakagawa T, Datta A, Kolodner RD: Multiple functions of MutS- and MutL-related heterocomplexes. Proc Natl Acad Sci U S A 1999, 96:14186–14188.

78 Lieber MR: The biochemistry and biologic significance of nonhomologous DNA end joining: an essential repair process in multicellular eukaryotes. Genes Cells 1999, 4:77–85.

79 •• Mathur R, Chowdhury MR, Singh G: Recent advances in chromosome breakage syndromes and their diagnosis. Indian Pediatr 2000, 37:615–625.
This article provides an account of DNA repair syndromes and their diverse etiology and clinical manifestations.

80 Glover TW: Instability at chromosomal fragile sites. Recent Results Cancer Res 1998, 154:185–199.

81 Jeggo PA: DNA breakage and repair. Adv Genet 1998, 38:185–218.

82 Vanasse GJ, Concannon P, Willerford DM: Regulated genomic instability and neoplasia in the lymphoid lineage. Blood 1999, 94:3997–4010.

83 Jeffreys AJ, Barber R, Bois P, et al.: Human minisatellites, repeat DNA instability and meiotic recombination. Electrophoresis 1998, 20:1665–1675.

84 Rothkamm K, Kuhne M, Jeggo PA, et al.: Radiation-induced genomic rearrangements formed by nonhomologous end-joining of DNA double-strand breaks. Cancer Res 2001, 61:3886–3893.

85 Johnson RD, Liu N, Jasin M: Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature 1999, 401:397–399.

86 •• Richardson C, Jasin M: Coupled homologous and nonhomologous repair of a double-strand break preserves genomic integrity in mammalian cells. Mol Cell Biol 2000, 20:9068–9075.
This study proposes the importance of homologous and nonhomologous repair pathways in maintaining genomic integrity.

87 Bishop AJ, Schiestl RH: Homologous recombination as a mechanism for genome rearrangements: environmental and genetic effects. Hum Mol Genet 2000, 9:2427–2434.

88 Ferguson DO, Sekiguchi JM, Chang S, et al.: The nonhomologous end-joining
•• pathway of DNA repair is required for genomic stability and the suppression of translocations. Proc Natl Acad Sci U S A 2000, 97:6630–6633.
Mutations in caretaker genes such as DNA ligase IV, *DNA-PKcs*, or *Ku70* display dramatic genomic instability ranging from chromosomal fragmentation to nonreciprocal translocation.

89 Difilippantonio MJ, Zhu J, Chen HT, et al.: DNA repair protein Ku80 sup-
•• presses chromosomal aberrations and malignant transformation. Nature 2000, 404:510–514.
Beautiful spectral karyotyping pictures of chromosomal abnormalities are displayed as chromosomal aberrations, including breakage and translocations in mouse cells deficient for *Ku80*.

90 Gao Y, Ferguson DO, Xie W, et al.: Interplay of p53 and DNA-repair protein
•• XRCC4 in tumorigenesis, genomic stability and development. Nature 2000, 404:897–900.
DNA NHEJ observed in *XRCC4*-deficient mice is exhibited as translocations in pro–B-cells and embryonic fibroblasts.

91 Hanahan D: Benefits of bad telomeres. Nature 2000, 406:573–574.
A brief account of the potential role of telomere defects in carcinogenesis.

92 Shay JW, Zou Y, Hiyama E, et al.: Telomerase and cancer. Hum Mol Genet 2001, 10:677–685.

93 Artandi SE, DePinho RA: Mice without telomerase: what can they teach us
•• about human cancer? Nat Med 2000, 6:852–855.
A working model for telomere attrition that can activate responses leading to chromosomal fusion, anaphase bridge formation, and breakage is described.

94 Zou H, McGarry TJ, Bernal T, et al.: Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 1999, 285:418–422.

95 Andersen SS: Spindle assembly and the art of regulating microtubule dynamics by MAPs and Stathmin/Op18. Trends Cell Biol 2000, 10:261–267.

96 Heald R: Motor function in the mitotic spindle. Cell 2000, 102:399–402.
••
An overview of the mechanics of motor function in the mitotic spindle apparatus.

97 Banks JD, Heald R: Chromosome movement: dynein-out at the kinetochore. Curr Biol 2001, 11:R128–R131.

98 Wittmann T, Hyman A, Desai A: The spindle: a dynamic assembly of microtubules and motors. Nat Cell Biol 2001, 3:E28–E34.

99 Carson DR, Christman MF: Evidence that replication fork components cata-
•• lyze establishment of cohesion between sister chromatids. Proc Natl Acad Sci U S A 2001, 98:8270–8275.
This article provides a nice account on sister chromatin cohesion proteins and their potential inactivation during the genesis of cancer.

100 Cahill DP, Lengauer C, Yu J, et al.: Mutations of mitotic checkpoint genes in
•• human cancers. Nature 1998, 392:300–303.
This study shows that chromosome instability is consistently associated with the loss of function of a mitotic checkpoint using mutational inactivation of *hBUB1*.

101 Orr-Weaver TL, Weinberg RA: A checkpoint on the road to cancer. Nature 1998, 392:223–224.

102 Haruki N, Saito H, Harano T, et al.: Molecular analysis of the mitotic checkpoint genes BUB1, BUBR1 and BUB3 in human lung cancers. Cancer Lett 2001, 162:201–205.

103 Jallepalli PV, Waizenegger IC, Bunz F, et al.: Securin is required for chromo-
•• somal stability in human cells. Cell 2001, 105:445–457.
The loss of hSecurin involved in the assembly of the anaphase-promoting complex leads to loss of chromosomes at high frequency, suggesting that hSecurin is essential for the maintenance of euploidy.

APPENDIX II-Resume

Sam Thiagalingam, M.S., Ph.D.

Sam Thiagalingam, Ph. D.

Assistant Professor
Genetics Program, Department of Medicine
Boston University School of Medicine
715 Albany Street, L320
Boston, MA 02118

Phone #: 617 638 6013
Fax #: 617 638 4275
E-mail: samthia@bu.edu

EDUCATION

University of Jaffna, Thirunelvely, Sri Lanka.	B.Sc. 1982	Biology
Bowling Green State University, Bowling Green, OH	M.S. 1986	Biology-Microbiology
The Johns Hopkins University, Baltimore, MD	Ph.D. 1992	Biochemistry
The Johns Hopkins Oncology Center, Baltimore, MD	1991 – 1995	PDF-Molecular Genetics/ Oncology

Graduate and Post-doctoral Advisors:

Doctoral Thesis Advisor: Dr. Lawrence Grossman, University Distinguished Service Professor of Biochemistry and Molecular Biology, The Johns Hopkins University, Baltimore, Maryland.

Post-doctoral Advisor: Dr. Bert Vogelstein, Clayton Professor of Oncology. The Johns Hopkins Oncology Center, Baltimore, Maryland.

PROFESSIONAL EXPERIENCE

1981 - 1982	Assistant Lecturer, Faculty of Science, University of Jaffna, Sri Lanka
1982 - 1984	Assistant Lecturer, Faculty of Science, Eastern University, Sri Lanka
1995 - 1998	Research Associate, The Johns Hopkins University School of Medicine
1998 -	Assistant Professor of Medicine, Boston University School of Medicine
1999 -	Assistant Professor of Pathology and Laboratory Medicine, Boston University School of Medicine
2002 -	Assistant Professor of Genetic and Genomics, Boston University School of Medicine

AWARDS AND HONORS

1983	Overseas Research Students Award, CVCP of the Universities of the U. K.
1988-1991	Post Certified Student Scholarship, The Johns Hopkins University
1992-1995	Amgen Post-doctoral Fellowship
1995	Oncology Fellow Research day Poster Award
1995-	Sterling Who's Who
1997-	Who's Who in the East
1999	American Lung Association Research Award
1999	American Cancer Society Institutional Research Grant Award
2000-2002	The Dolphin Trust Investigator (New Investigator Award), The Medical Foundation, MA
2001	Burroughs Wellcome Fund New Investigator in Toxicological Sciences Award (Finalist)
2001-2005	Career Development Award, Department of Defense BCRP, U.S. Army MRMC

PROFESSIONAL ORGANIZATIONS

American Association for Cancer Research	-	Member
American Society of Human Genetics	-	Member
American Society for Biochemistry and Molecular Biology	-	Member
American Association for Advancement of Science	-	Member

EDITORIAL RESPONSIBILITIES

Editorial boards: Cancer Biology and Therapy, Journal of Molecular Biology and Biotechnology.

Ad hoc reviewer: Cancer Research, Cell Growth & Differentiation, Cancer Detection and Prevention, Cancer Epidemiology, Biomarkers and Prevention, Clinical Genetics, The American Journal of Physiology - Cell Physiology, Physiological Genomics and Proceedings of the National Academy of Sciences, USA.

NIH GRANT REVIEW

Ad hoc reviewer: R03 Awards, NIDA, NIH

BIBILIOGRAPHY

Number of citations (Institute for Scientific Information) as of 09/18/02: 2,571

Ponte, J. F., M. Levy, J. S. Brody and **S. Thiagalingam**. 2002. Modulation of p53 by hBUB1 is involved in the mitotic checkpoint. *Manuscript submitted.*

Cheng, K-h., H. J. Lee, J. F. Ponte and **S. Thiagalingam.** 2002. Identification of alternate targets for the inactivation of Smad signaling in cancer using a novel method: Targeted Expressed Gene Display. *Manuscript submitted.*

Ponte, J. F., K-h.Cheng, J. S. Brody and **S. Thiagalingam.** 2002. Detection of genetic damage in pre-neoplastic cells: A non-invasive diagnostic procedure. *Manuscript in preparation.*

Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGF-β and its *Smad* connection to cancer. *Current Genomics* **3**: 449-476.

Thiagalingam, S., R. L. Foy, K-h.Cheng, H. J. Lee, A. Thiagalingam, and J. F. Ponte. 2002. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. *Current Opinion in Oncology* **14(1)**: 65-72.

Thiagalingam, S., S. Laken, J. K. V. Willson, S. Markowitz, K. W. Kinzler, B. Vogelstein and C. Lengauer. 2001. The mechanisms underlying losses of heterozygosity in human colorectal cancers. *Proc. Natl. Acad. Sci.* USA. 98: 2698-2702.

Thiagalingam, S. 2001. Molecular detection of Smad4/Smad2 alterations in colorectal tumors: Colorectal Cancer Methods and Protocols. In *Methods in Molecular Medicine*. S. M. Powell (Ed) Humana Press Inc., New Jersey, **50**: 149-165.

Fink, S. P., S. E. Swindler, R. S. Brady, J. D. Lutterbaugh, J. Massague, **S. Thiagalingam,** K. W. Kinzler, B. Vogelstein. J. K. V. Willson, and S. Markowitz. 2000. TGF-β induced inhibition in a Smad4

mutant colon adenoma cell line. *Cancer Res.* **61**: 256-260.

Thiagalingam, S., Sau, K., Cheng, K-h., Foy, R. L., and J.F. Ponte. 2000. Identification of upstream kinase regulators of p53 function. *FASEB Journal* **14(8):** 1331

Grady, W. M., L. L. Myeroff, S. E. Swindler, A. Rajput, **S. Thiagalingam**, J. D. Lutterbaugh, A. Neumann, M. G. Brattain, J. Chang, S.-J. Kim, K. W. Kinzler, B. Vogelstein. J. K. V. Willson, and S. Markowitz. 1999. Mutational inactivation of TGF-β receptor type II in microsatellite stable colon cancers. *Cancer Res.* 59: 320-324.

Hermeking, H, C. Lengauer, K. Polyak, T.-C. He, L. Zhang, **S. Thiagalingam**, K. W. Kinzler and B. Vogelstein. 1997. 14-3-3-σ mediates a p53 induced G2 arrest. *Molecular Cell* 1: 3-11.

He, T.-C, L. T.Costa and **S. Thiagalingam.** 1997. Homeosis and Polyposis: A Tale from the Mouse. *BioEssays* **19**: 551- 555.

Riggins, J. G., K. W. Kinzler, B. Vogelstein and **S. Thiagalingam.** 1997. Frequency of *Smad* gene mutations in human cancers. *Cancer Res.* 57: 2578-2580.

Riggins, J. G., **S. Thiagalingam**, E. Rozenblum, C. L. Weinstein, S. E. Kern, S. R. Hamilton, J. K. V. Willson, S. Markowitz, K. W. Kinzler and B. Vogelstein. 1996. *MAD*-related genes in the human. *Nature Genet.* **13**: 347-349.

Thiagalingam, S., C. Lengauer, F. S. Leach, M. Schutte, S. A. Hahn, J. Overhauser, J. K. V. Willson, S. Markowitz, S. R. Hamilton, S. E. Kern, K. W. Kinzler and B. Vogelstein. 1996. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nature Genet.* **13**: 343-346.

Thiagalingam, S., N. A. Lisitsyn, M. Hamaguchi, M. H. Wigler, J. K. V. Willson, S. D. Markowitz, F. S. Leach, K. W. Kinzler and B. Vogelstein. 1996. Evaluation of *FHIT* gene in colorectal cancers. *Cancer Res.* **56**: 2936-2939.

Thiagalingam, S., K. W. Kinzler and B. Vogelstein. 1995. *PAK1*, a gene that can regulate p53 activity in yeast. *Proc. Natl. Acad. Sci.* USA. **92**: 6062-6066.

Tokino, T., **S. Thiagalingam**, W. S. El-Deiry, T. Waldman, K. W.Kinzler, and B. Vogelstein.1994. *p53*-tagged sites from human genomic DNA. *Human Mol. Genet.* **3**: 1537-1542.

Pietenpol, J. A., T. Tokino, **S. Thiagalingam**, W. S. El-Deiry, K. W. Kinzler, and B. Vogelstein. 1994. Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl. Acad. Sci.* **91**:1998-2002.

Grossman, L., B. Ahn, L. Claassen, E. Hildebrand, S. Mazur, E. Oh, T. Seeley, **S. Thiagalingam**, and J. T. Wang. 1994. Nucleotide excision-repair - A tracking mechanism in search of damage. *J. Cell. Biochem.* Supplement **18C**: p129.

Oliner, J. D., J. A. Pietenpol, **S. Thiagalingam**, K. W. Kinzler, and Vogelstein, B. 1993. Oncoprotein MDM2 conceals the activation domain of p53. *Nature* **362**: 857-862

Grossman, L., and **S. Thiagalingam.** 1993. Nucleotide excision repair, a tracking mechanism in search of damage. *J. Biol. Chem.* **268**: 16871-16874.

Grossman, L., B. Ahn, L. Claassen, E. Hildebrand, S. Mazur, E. Oh, T. Seeley, **S. Thiagalingam**, and J. T. Wang. 1993. Nucleotide Excision Repair - A study in vectorial movement. *J. Cell. Biochem.* Supplement **17C**: p148.

Thiagalingam, S., and L. Grossman. 1993. The multiple roles for ATP in the *Escherichia coli* UvrABC catalyzed incision reaction. *J. Biol. Chem.* **268**: 18382-18389.

Thiagalingam, S., and T. Yang. 1993. Purification and characterization of NADH dehydrogenase from *Bacillus megaterium. Can. J. Microbiol.* **39**: 826-833.

Kern, S. E., J. A. Pietenpol, **S. Thiagalingam**, A. Seymour, K. W. Kinzler and B. Vogelstein. 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* **256**: 827-830.

Thiagalingam, S., and L. Grossman. 1991. Both ATPase sites of *Escherichia coli* UvrA have functional roles in nucleotide excision repair. *J. Biol. Chem.* **268**: 18382-18389.

Thiagalingam, S., and L. Grossman. 1991. Functional roles of the ATPase sites of *Escherichia coli* UvrAB in nucleotide excision repair. *J. Cell. Biochem.* Supplement **15D**: p110.

Oh, E. Y., L. Claassen, **S. Thiagalingam**, S. Mazur and L. Grossman. 1989. ATPase activity of UvrA and UvrAB protein complexes of the *Escherichia coli* UvrABC endonuclease. *Nucleic Acids Res.* **17**: 4145-4159.

Thiagalingam, S., Oh, E. Y., Mazur, S., and L. Grossman. 1988. Allosteric interactions between nucleotide and DNA binding sites in the *E. coli* UvrA protein. *J. Cell. Biochem.* Supplement **12A**: p278.

Thiagalingam, S., and T. Yang. 1986. NADH dehydrogenase and NADH oxidase in *Bacillus megaterium. Curr. Microbiol.* **14**: 217-220.

SYNERGISTIC ACTIVITIES

(i) Developed a double selection genetic assay to identify genes upstream of p53 function.

(ii) Validated the double selection genetic assay for the identification of human p53 regulatory genes and isolated established regulators as well as previously unknown novel regulators such as hBUB1.

(iii) One of the two major contributors to the discovery of five novel Smad genes (i.e., Smad1, Smad2, Smad3, Smad5, Smad6).

(iv) Elucidated the molecular mechanism of loss of heterozygosity (LOH) in cancer with the use of molecular genetics and cytogenetics. These studies also confirmed that LOH is a reliable predictor for the genome localization of tumor suppressor genes.

(v) Developed a novel method known as Targeted Expressed Gene Display (TEGD) for the discovery and/or analyses of a family of genes.

(vi) Actively participates in training and educating the future generation of cancer researchers, educators and medical & public health practitioners by providing knowledge on the molecular basis of cancer and the application of genomic state of the art methodologies.